Title: DIAGNOSIS OF GLUTEN-INDUCED AUTOIMMUNE DISEASES

Abstract: The invention relates to selective diagnosis of gluten-induced autoimmune diseases by binding assays utilizing the main celiac epitope present on proteins of the transglutaminase family.
DIAGNOSIS OF GLUTEN-INDUCED AUTOIMMUNE DISEASES

FIELD OF THE INVENTION

The invention relates to selective diagnosis of gluten-induced autoimmune diseases by binding assays utilizing the main celiac epitope present on proteins of the transglutaminase family.

BACKGROUND ART

Celiac disease (also known as celiac disease, non-tropical sprue or gluten-sensitive enteropathy) is a unique autoimmune disorder for which the environmental trigger of the disease is known. The disorder develops among genetically predisposed persons with the human leucocyte antigen (HLA) DQ2 or DQ8 background and is characterized by chronic bowel inflammation, malabsorption and production of autoantibodies as a result of gluten consumption. Gluten also can induce in addition to the enteropathy an itchy and blistering skin rash, and in this case, the disease is called dermatitis herpetiformis. The disease can successfully be treated by elimination of gluten from the patients’ diet, but for a sustained response this treatment should be followed life-long.

Celiac disease is precipitated by the ingestion of the gluten fraction of wheat, rye and barley. Gliadin is the alcohol-soluble part of gluten and contains a mixture of peptides that were shown to be toxic for the patients. A 33-mer, α-gliadin-derived peptide was identified as the most immunogenic part of gliadin, which is resistant to gastric, pancreatic and brush-border membrane protease-digestion and can pass bowel epithelium to provoke immune response in the lamina propria [Shan L et al, Science. 2002;297:2275-9.] Chronic inflammatory cell infiltration, villous atrophy and crypt hyperplasia in the upper small intestine are characteristic features of the disease, while it can also cause extraintestinal symptoms such as anemia, neurological disorders or the skin disease called dermatitis herpetiformis. The disease primarily occurs in Western populations where wheat is the basic food (Europe, USA, Australia), but recently it has appeared in the Middle East, South America, North Africa and Asia too. The disease was found in 1.0 % of Finnish schoolchildren [Maki M et al, N Engl J Med. 2003;348(25):25 17-24.,] in 0.75% of the USA "not-at-risk" groups [Fasano A et al, Arch Intern Med. 2003;163(3):286-92.] and in 1.4% of Hungarian 6 years old children by screening [Korponay-Szabo IR et al, BMJ. 2007;335(7632):1244-7].

The disease has a wide range of various clinical manifestations such as diarrhea, abdominal distension, anemia, weight loss, retarded growth, skin or neurologic symptoms and can be diagnosed at any age. As a result of the lack of proper absorption, deficiencies of vitamins and minerals, vitamin B₁₂, vitamin K, folic acid, iron and calcium, can occur. Many patients may have only subtle symptoms and in such cases the proper diagnosis is often delayed. Untreated celiac disease has serious complications like adenocarcinoma (with a risk almost twice compared to the general population), T-cell lymphoma (with approximately fifty-fold risk) and osteoporosis.
The current diagnostic criteria of celiac disease were established by the European Society for Pediatric Gastroenterology and Nutrition and require histological examination of a biopsy sample from the upper part of the small bowel (duodenal biopsy) featuring intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy plus a favourable response to a gluten-free diet also should be observed. It also supports the diagnosis of celiac disease if the patient displays in blood certain autoantibodies that can bind to normal tissue sections along the connective tissue sheets surrounding smooth muscle cells (endomysial antibody) or along reticulin fibers (R, reticulin antibody binding pattern) [Green PH, Cellier C. N Engl J Med. 2007;357(17):1731-43.].

The disease is heavily underdiagnosed and only two of ten patients are clinically recognized. The main explanation for this is that not all of the patients develop clinical manifestations (silent disease) and some may even have normal mucosal morphology for a long time [Maki M, Collin P, Lancet. 1997;349(9067):1755-9.]. However, they are often positive for reticulin and endomysial tissue autoantibodies with a high density of intraepithelial lymphocytes, or with positive celiac-like intestinal antibody pattern. This subgroup of the disease called latent or potential celiac disease is a diagnostic challenge and these patients may present villous atrophy and crypt hyperplasia in later years or may develop severe - sometimes irreversible - damage in other organs (gluten ataxia, cardiomyopathy, diabetes mellitus, hypothyreosis). The diagnosis in patients with a latent phase is difficult, as they initially often do not fulfill conventional diagnostic criteria and they need a long follow-up time. Antibody levels may fluctuate over time, and presently there are no good tools to exactly predict which subjects will deteriorate in the near future [Simell S et al, Scand J Gastroenterol. 2005;40(10):182-91.].

Current diagnostic criteria based on the established villous damage in the small intestine are thus insufficient and need reformulation [Kaukinen K et al, Dig Dis Sci. 2001;46:879-87.] In addition, small bowel biopsy is an unpleasant and invasive diagnostic procedure and substitution of biopsy-based diagnostic criteria by antibody positivity would have several practical advantages, and also the possibility to include subjects with early disease and formulate treatment indication in time.

The need for less complicated and better tolerable initial diagnostic tools has long been recognized. Serologic tests based on the detection of circulating antibodies in the patients’ blood against gliadin, reticulin, endomysium and type-2 transglutaminase (TG2) are increasingly used. They are also useful for finding patients with mild symptoms or atypical clinical manifestations and for the screening of first degree relatives or risk groups. The main risk groups consist of patients with diabetes mellitus, Down’s syndrome, selective IgA deficiency and various autoimmune disorders like autoimmune thyroid disease, Sjogren’s syndrome, lupus erythematosus, autoimmune liver disease, alopecia, glomerular disease and heart disease which often co-exist with celiac disease [Green PH, Cellier C. N Engl J Med. 2007;357(17):1731-43.].

Antigliadin antibodies do not show enough sensitivity or specificity for celiac disease, despite this, a test based on deamidated gliadin peptides that have structural similarity with TG2 is relatively efficient [Volta U et al, Dig Dis Sci. 2008;53(6):1582-8.; Korponay-Szabo IR et al, J Pediatr...
Gastroenterol Nutr. 2008;46(3):253-61. Monitoring endomysial IgA antibodies using indirect immunofluorescence assays can provide a high accuracy, since these antibodies are highly specific markers of the disease. However, evaluation of the endomysial antibody test is largely observer-dependent and requires highly skilled and trained personnel and thus it is not widely available in each medical setting. Selective IgA deficiency is a common feature in celiac patients (1 in 40 cases) which makes very difficult to recognize patients by the conventional endomysial antibody test. In these cases, IgG class anti-TG2 autoantibodies should be detected [Korponay-Szabo I et al, Gut. 2003;52(II): 1567-71]. Further, the endomysial test cannot differentiate between the overt and latent forms of the disease [Green PH, Cellier C, N Engl J Med. 2007;357(17):1731-43.]. Thus, there was a need in the art for reliable and more easy-to-use tests.

TG2 (also known as tissue, erythrocyte or cellular transglutaminase) was found to be the main self autoantigen and a target for endomysial and reticulin antibodies in celiac disease. The enzyme is a member of the transglutaminase superfamily, which enzymes catalyze acyl-transfer reaction between γ-carboxamide group of a glutamine and the ε-amino group of a lysine residue -covalent protein crosslinking- in a Ca²⁺-dependent manner (EC 2.3.2.13). Besides this TG2 can catalyze incorporation of amines into proteins, site-specific deamidation, it has isopeptidase activity and takes part in transmembrane signaling via its GTPase activity. TG2 can externalize from the cells where it is complexed with integrins and fibronectin, promotes the remodelling and assembly of the extracellular matrix and mediates cell-matrix interactions. In this way TG2 takes part in wound healing and angiogenesis. The enzyme can translocate to the nucleus and with crosslinking of histones and retinoblastoma proteins it also can regulate gene expression. TG2 is induced and activated during apoptosis; moreover the lack of the enzyme affects the engulfment of apoptotic cells by macrophages [Fesus L, Piacentini M, Trends Biochem Sci. 2002;27(10):534-9.].

TG2 is a ubiquitous enzyme, it can be found all over the body, intra- and also extracellularly. After the identification of the main autoantigen TG2, numerous enzyme-linked immunoassays (ELISA) were developed with guinea pig liver TG2 or recombinant human TG2 as antigen [Schuppan D et al, 1998; WO 98/03872.; Powell M et al, 2002; WO 02/068616 A2.]. Further, extracellular matrix rich in TG2 produced by cell lines can also be used as antigen in diagnostic tests. These assays are less expensive than endomysial antibody tests and their sensitivity and specificity is greater than 90% in the case of human TG2 antigen. Moreover, efficient rapid antibody detection was described by utilizing the natural self TG2 in the red blood cells of patient blood samples [Maki M et al, 2002; WO 02/086509].

In some patients, and particularly in those with dermatitis herpetiformis, antibodies against a homologous skin transglutaminase protein (TG3) were also described, that could be used as well for diagnostic purposes (Paulsson M et al., 2001, WO 01/001 133). Hadjivassiliou et al. [Hadjivassiliou et al, Ann Neurol. 2008;64:332-43] suggested that antibodies against transglutaminase 6 (TG6) can serve as a marker of a gluten induced autoimmune disease, in addition to human leukocyte antigen type and detection of anti-gliadin and anti-transglutaminase 2
antibodies, to identify a subgroup of patients with gluten sensitivity who may be at risk for development of neurological disease. They observed that TG6 IgG and IgA response is prevalent in gluten ataxia, independent of intestinal involvement.

In fact, several studies based on TG2-based tests of the art described false negative IgA TG2 results with IgA endomysium antibody positivity and false positive IgA TG2 results in the absence of IgA endomysium positivity [Wong RC et al, J Clin Pathol. 2002;55(7):488-94.]. False positive TG2 antibody positive results are relatively common in the clinical settings [Lock RJ et al, Eur J Gastroenterol Hepatol. 2004;16(5):467-70.; Green PH, Cellier C, N Engl J Med. 2007;357(17):1731-43.] and this severely restricts the use of TG2 antibody positivity as the sole diagnostic test. Particularly, patients with other autoimmune diseases, tumors, cardiac failure, neurological disorders, psoriasis and liver diseases may exhibit low levels of antibodies reacting with TG2. This is not surprising, because these tissues contain relatively high amounts of TG2 which can be liberated by any kind of tissue damage that can induce non-specific autoantibodies against TG2 and this antibody positivity may be irrelated to celiac disease.

In contrast, celiac-specific antibodies are induced in response to gluten probably by a hapten-carrier mechanism [Solid LM et al, Gut. 1997;41(6):851-2.], and recognize TG2 in the form as it is bound to the surface of fibronectin in muscular tissue section substrates used for the endomysial antibody detection by immunofluorescence. However, even the endomysial antibody test cannot distinguish between anti-TG2 antibodies of celiac patients and other TG2 antibodies experimentally induced in mice [Korponay-Szabo IR et al, J Pediatr Gastroenterol Nutr. 2008;46(3):253-61.]. Further, in an attempt to produce more celiac-specific diagnostic tests, TG2 complexed with gliadin peptides was also used as antigen, but this gave even less reliable results than TG2 alone [Rajadhyaksha M et al, WO 01/29090 A1]. Thus a verification immunoassay differently recognizing antibodies directed against the celiac and other TG2 epitopes could be exquisitely important for the simplification of the diagnostic process in future.

Thus, there is still a need to develop a reliable and relatively convenient diagnostic method which is specific for a gluten-induced autoimmune disease or preferably, celiac disease. In particular, there is still a need in the art to develop a diagnostic assay which could identify latent form of the disease, and/or which is applicable in children or in cases when symptoms do not provide a decisive answer. Moreover, replacing diagnosis based on jejunal biopsy would also be desirable in terms of cost and patient comfort.

In fact, several efforts have been made in the art to identify the main binding epitopes of the celiac autoantibodies. However, no such unique epitope has been found so far and it is also not clear whether only one or several such epitopes exist. Previous studies [Seissler J et al, Clin Exp Immunol. 2001;125(2):216-21.; Sblettero D et al, Eur J Biochem. 2002;269(21):5 175-81.; Nakachi K et al, J Autoimmun. 2004;22(1):53-63.], in which TG2 fragments were applied, indicated that the C-terminal domain may harbor important binding sites. However, some patient samples also recognized the N-terminal or the core domain, so that it was concluded that the antibody response may be dispersed and
variable according to the subjects. Further, a difference was noted between young female celiac patients and those with other clinical manifestations [Tiberti C et al, Clin Immunol. 2003;109(3):318-24.]. In another study [Byrne G et al, Gut. 2007;56(3):336-41.], the catalytic triad was suggested as the main binding site, as mutation of these three amino acids resulted in a protein to which patient antibodies bound poorly. Again in this study, the specificity of IgA and IgG class celiac antibodies seemed to be different. However, these studies did not take into account the three-dimensional structure of the protein which might severely be deranged by gross deletions, particularly by those affecting the core domain or the catalytic triad. Further studies conducted by TG2 fragments displayed on the surface of phages also indicated that the conformation of the protein might have a high importance to form a functional binding site for the celiac antibodies, as it was not possible to identify the epitopes of celiac patient derived monoclonal antibodies by these means either [Di Niro R et al, Biochem J. 2005;388(3):889-94.].

TG2 is not only a main autoantigen in celiac disease and thus an important target molecule in the diagnosis, but it is also involved in the pathogenesis of the disease.

Increased TG2 expression and activity were detected in the duodenal biopsies of celiac patients compared to healthy donors and gliadin peptides were found to be good substrates for TG2. According to the present hypothesis, gliadin-TG2 complexes can bind to TG2-specific B cells, where both gliadin and TG2 fragments can be presented on DQ2. The presented gliadin-DQ2 complexes can activate the gliadin-specific T-cells, which can provide help for the TG2-specific B cells to produce anti-TG2 antibodies by a hapten-carrier mechanism [Sollid LM et al, Gut. 1997;41(6):851-2.]. Celiac disease is conventionally regarded as a mainly T-cell mediated disorder where the role of celiac antibodies is debated. Interestingly, celiac antibodies do not block the cross-linking activity of TG2 [Dieterich W et al, Gut. 2003;52(II):1562-6.; Roth EB et al, Clin Exp Rheumatol. 2006;24(1):12-8.], instead, they may even enhance the transamidation and deamidation processes presumably by stabilising the enzyme's conformation [Kiraly R et al, J Autoimmun. 2006;26(4):278-87.]. In this way, TG2 will process more gliadin, which could enhance the immune response ending in more and more antibody production and driving the disease process further. Proposals in the art that the epitope is conformational and multiple domains are involved apparently contradict this finding.

Several lines of new evidence suggest that autoantibodies against TG2 also do have pathogenic role in the development of the disease. Presence of these antibodies is a uniform feature in all patients and if not detectable in the circulation, these antibodies can be found deposited in various tissues. Tissues damage has been related to the in vivo binding of antibodies and antibodies bound to extracellular TG2 were found in situ in diseased organs like small bowel, liver, muscles, kidney, brain etc also in the absence of positivity for the antibodies in serum samples [Korponay-Szabo IR et al, Gut. 2004;53(5):641-8.]. These antibodies are functional as they can bind externally added recombinant TG2 and they appear in tissues already before the villous atrophy develops [Salmi TT et al, Gut. 2006;55(12):1746-53.; Salmi TT et al, Aliment Pharmacol Ther. 2006;24(3):541-52.].

The present inventors unexpectedly found that a main celiac epitope can be localized on transglutaminases which function as autoantigens in gluten induced autoimmune diseases. Surprisingly, improved diagnostic methods can be provided based on engineered transglutaminases having a folded beta-sandwich and core domains, by utilizing binding pattern of celiac autoantibodies and thereby eliminating false positive results. Moreover, further improved diagnostic method can be provided by using compounds specifically binding to the main celiac epitope and being capable of displacing or displaced by celiac patient autoantibodies.

**BRIEF DESCRIPTION OF THE INVENTION**

According to a first aspect the invention relates to a diagnostic method for diagnosis of a gluten-induced autoimmune disease in a subject comprising the steps of

i) taking a biological sample from or providing a biological sample taken from a subject, said sample containing autoantibodies of said subject, and optionally isolating autoantibodies from said sample,

ii) contacting the autoantibodies of said sample with

- a reference protein belonging to the transglutaminase family (a TG family protein) and having an integral main celiac epitope,

- at least one test protein belonging to the transglutaminase family, in which the side chain and/or spatial position of at least one surface amino acid residue contributing to the main celiac epitope is altered as compared to the reference protein in which the main celiac epitope is integral, so that the binding level of an antibody known to be capable of binding to said main celiac epitope is impaired in said test protein

wherein the said at least one surface amino acid residue of the main celiac epitope comprises or is selected from

- one or more surface amino acid residues of the first alpha helix of the core domain, preferably a surface amino acid residue selected from the first four, three, or two amino acid residues of said alpha helix, more preferably the first amino acid residue of said alpha helix, and/or

- one or more surface amino acid residues of the first alpha helix of the beta-sandwich domain and one or more surface amino acid residues of the conserved HisHisThr motif of the beta sandwich domain, preferably a surface amino acid residue selected from the last six, five, four or three amino acid residues of said
first alpha helix of the beta-sandwich domain and amino acid residues of the HisHisThr motif, more preferably the sixth amino acid residue of said helix and/or the first amino acid of said HisHisThr motif, and wherein the core domain has a folded three dimensional structure, preferably a native folded three dimensional structure, and the beta-sandwich domain has a folded three dimensional structure, preferably a native folded three dimensional structure, preferably the fold of these domains is essentially integral or is maintained,

iii) assessing a binding property of the autoantibodies to the reference protein and to the at least one test protein,

wherein if the binding of autoantibodies to the test protein is impaired as compared to the reference protein, this fact is considered as indicative of a gluten-induced autoimmune disease in said subject. Preferably, when a side chain is altered, the side chain geometry, side chain size, side chain functional group or charge thereof is altered.

Preferably the binding property is assessed by assessing the binding level or by assessing binding kinetic rates or parameters, e.g. those of association and/or dissociation.

In a preferred embodiment in step iii) of the diagnostic method the binding is assessed by measuring binding level of the autoantibodies to the test protein and to the reference protein, wherein if the binding level of the autoantibodies to the reference protein exceeds a predetermined threshold value and if the binding level of the autoantibodies to the test protein is significantly lower than to the reference protein or preferably reduced by at least a predetermined ratio, preferably by at least 30%, more preferably at least 40%, even more preferably at least 50% as compared to the reference protein, this fact is considered as indicative of a gluten-induced autoimmune disease in said subject.

Setting a predetermined threshold (or cut-off) is a routine optimisation process and is well within the skills of a person skilled in the art of setting assay conditions. A suitable cut-off for declaring binding molecules, e.g. antibodies as reactive with a TG family protein, e.g. a reference TG family protein can be established for example by a receiver operating characteristic curve (ROC) performed with known celiac and non-celiac samples, as well known in the art.

In an embodiment, the predetermined threshold is set to 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 15%, 16%, 17%, 18%, 19% or 20% of the maximal reference value, e.g. of the level of 100% binding.

Preferably, the first alpha helix of the beta-sandwich domain spans from an amino acid residue which is in eight amino acid distance in the N-terminal direction from the first His of the HisHisThr motif of the beta sandwich domain to said first His of the HisHisThr motif of the beta sandwich domain.

Preferably, the last five amino acids of the first alpha helix of the beta-sandwich domain spans from the conserved Asn of the first alpha helix of the beta-sandwich domain to the first His of the conserved HisHisThr motif of the beta-sandwich domain and preferably the last six, five, four or three amino acid residues of said helix are calculated accordingly.
Thus, preferably the sixth amino acid residue of said helix is the second amino acid in amino terminal direction from the conserved HisHisThr motif of the beta-sandwich domain, or the second amino acid in carboxy terminal direction from the conserved Asn of the first alpha helix of the b-sandwich domain.

Preferably, the sixth amino acid of the first alpha helix of the beta-sandwich domain is Arg 19 in human TG2 or a corresponding amino acid in the sequence of a protein of the transglutaminase family having a folded beta-sandwich domain and a folded core domain; and the first His of the HisHisThr motif is His22 in human TG2 or a corresponding amino acid in the sequence of a protein of the transglutaminase family having a folded beta-sandwich domain and a folded core domain.

Preferably, the first alpha helix of the core domain spans
- from the fifth amino acid in amino terminal direction from the conserved GluTyrXxx motif (wherein Xxx is an apolar amino acid residue, preferably Val or He or Leu) of the first alpha helix of the core domain or
- from the third amino acid in amino terminal direction from the conserved Arg of the first alpha helix of the core domain of human transglutaminases
- to at least the Glu or Tyr of said GluTyrXxx motif.

Thus, the first or the first two, three or four amino acid residues of the first alpha helix of the core domain is/are calculated accordingly.

More preferably, the first amino acid residue of said alpha helix is the fifth amino acid in amino terminal direction from the conserved GluTyrXxx motif of the first alpha helix of the core domain or the third amino acid in amino terminal direction from the conserved Arg of the first alpha helix of the core domain of the majority of human transglutaminases.

In a preferred embodiment the spatial position or the side chain geometry of

a) at least a surface amino acid residue of the first alpha helix of the core domain, preferably a surface amino acid residue selected from the first four, three, or two amino acid residues of said alpha helix, more preferably the first amino acid residue of said alpha helix, and

b) at least a surface amino acid residue of the first alpha helix of the beta-sandwich domain and the conserved HisHisThr motif of the beta sandwich domain, preferably a surface amino acid residue selected from the last six, five, four or three amino acid residues and the HisHisThr motif, more preferably the sixth amino acid residue of said helix,

are altered relative to each other.

In a further embodiment a mutation is carried out in a part of the protein of the transglutaminase family which results in the dislocation of or an altered spatial position of any of the amino acids defined herein as an amino acid contributing to the main celiac epitope or an SSE carrying said protein. In certain embodiments larger parts of the alpha helices are dislocated, e.g. the alpha helices are disrupted or tilted.

In the diagnostic method of the present invention preferably
the first amino acid residue of said first alpha helix of the core domain is an amino acid residue corresponding to or equivalent to Glu153 numbered according to the amino acid numbering of the full length human TG2 based on amino acid sequence alignment, and/or

the sixth amino acid residue(s) of said first alpha helix of the beta-sandwich domain is an amino acid residue corresponding to or equivalent to Arg19 numbered according to the amino acid numbering of the full length human TG2 based on amino acid sequence alignment, and/or

the first amino acid of the HisHisThr motif said first alpha helix of the beta-sandwich domain is an amino acid residue corresponding to or equivalent to His22 numbered according to the amino acid numbering of the full length human TG2 based on amino acid sequence alignment.

In a preferred embodiment of the diagnostic method according to the present invention in said TG family test protein the side chain or spatial position of at least one further amino acid is altered, e.g. one or more further amino acid is mutated, as compared to the reference protein in which the main celiac epitope is integral,

wherein preferably said further amino acid is selected from the group of amino acid residues corresponding to or equivalent to Arg 151, Glu 153, Glu 154, Arg156, Arg 19, His22, Val431, Arg433, Glu435, Met659, Leu661 numbered according to the amino acid numbering of the full length human TG2 based on multiple sequence alignment and wherein preferably said alteration effects celiac antibody binding.

In a further preferred embodiment the spatial position of any of the following amino acid residues is altered due to the alteration of one or more further amino acid as compared to the reference protein:

a surface amino acid residue selected from the first four, three, or two amino acid residues of said alpha helix, more preferably the first amino acid residue of said alpha helix, and/or

a surface amino acid residue selected from the last six, five, four or three amino acid residues of said first alpha helix of the beta-sandwich domain and amino acid residues of the HisHisThr motif, more preferably the sixth amino acid residue of said helix and/or the first amino acid of said HisHisThr motif.

In a preferred variant of this embodiment the said further alteration is a mutation as compared to the reference protein in one or more amino acid residues selected from the group of amino acid residues corresponding to or equivalent to the following amino acids of the full length human TG2 based on multiple sequence alignment: Glu158, Tyr160, Val161, His23, Thr24, or an alteration in the N-terminal portion of the beta-sandwich domain, e.g. a deletion or substitution of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, amino acid residues calculated from the sixth residuee of the first alpha helix of the beta-sandwich domain.

Preferably, the test protein is a mutant TG2 and the alteration of said one or more amino acids is a mutation selected from the following group:

a mutation of

Arg19, e.g. R19S, R19K, R19Q
Glu54, e.g. E154S, E154T, E154Y, E154K, E154R, E154Q, E154N,
Glu58, e.g. E158S, E158Q, ...

and optionally

Ala24, and celiac disease

A skilled person will understand that if the effect of the mutation or alteration in the side chain is small, e.g. if a conservative substitution is made, it may be expedient to have more than one amino acid residues mutated.

In a further embodiment of the present invention the autoantibodies or a panel of autoantibodies are(is) isolated before contacting them with the test protein and, if desired, with the reference protein.

In a further aspect the invention relates to a diagnostic kit for the diagnosis of a gluten-induced autoimmune disease in a subject comprising

a) a test protein belonging to the transglutaminase family, in which the side chain and/or spatial position of at least one surface amino acid residue contributing to the main celiac epitope is altered as compared to the reference protein in which the main celiac epitope is integral,

wherein the said at least one surface amino acid residue of the main celiac epitope comprises or is selected from

a surface amino acid residue of the first alpha helix of the core domain, preferably a surface amino acid residue selected from the first four, three, or two amino acid residues of said alpha helix, more preferably the first amino acid residue of said alpha helix, and/or

a surface amino acid residue of the first alpha helix of the beta-sandwich domain and a surface amino acid residue of the conserved HisHisThr motif of the beta sandwich domain, preferably a surface amino acid residue selected from the last six, five, four or three amino acid residues of said first alpha helix of the beta-sandwich domain and amino acid residues of the HisHisThr motif, more preferably the sixth amino acid residue of said helix and/or the first amino acid of said HisHisThr motif,

and wherein the core domain has a folded three dimensional structure, preferably a native folded three dimensional structure, and

b) a reference protein belonging to the transglutaminase family, said protein having an integral main celiac epitope or at least a medium carrying instructions for providing and using said reference protein belonging to the transglutaminase family;

and optionally
means for taking a biological sample from a subject, said sample containing autoantibodies of said subject and/or means for isolating autoantibodies from said sample, and/or means for assessing the level of binding and/or kinetics of the autoantibodies to the proteins.

In the diagnostic kit of the invention any test protein and/or reference protein as defined herein can be applied.

In a preferred embodiment

a) the test protein is a mutant transglutaminase (TG), preferably a mutant TG2, TG3 or TG6, and
b) the reference protein is a wild type TG, preferably a wild type TG2, TG3 or TG6.

In a preferred embodiment the means for assessing the level of binding comprises a plate e.g. a microtiter plate having wells wherein a first part of the wells are coated with the reference protein and a second part of the wells are coated with the at least one test protein.

In an embodiment the wells of the plate are also coated with fibronectin.

In a further aspect the invention relates to a diagnostic method for the diagnosis of a gluten-induced autoimmune disease in a subject comprising the steps of

i) taking a biological sample or providing a biological sample taken from said subject, said sample containing autoantibodies of said subject and optionally isolating autoantibodies from said sample,
ii) contacting autoantibodies of said sample with a protein belonging to the transglutaminase family, said protein having an integral main celiac epitope and
iii) assessing the level of binding of the autoantibodies to the TG family protein both in the absence of and in the presence of a test compound, said test compound known to be capable of binding to the main celiac epitope,

wherein if the binding level of the autoantibodies in the absence of the test compound exceeds a predetermined threshold value and if the binding level of the autoantibodies to the reference protein in the presence of the test compound is significantly lower as compared to the binding level of the autoantibodies in the absence of said test antibody, this fact is considered as indicative of a gluten-induced autoimmune diseases in said subject. Preferably the binding level of the autoantibodies to the reference protein in the presence of the test compound is reduced by at least a predetermined ratio, preferably by at least at least 40%, preferably at least 50%, more preferably at least 60%, 70% or 80%, corresponding to a binding level wherein the remaining binding is not higher than 60%, preferably not higher than 50%, more preferably not higher than 40%, 30% or 20%, respectively.

The test compound, therefore, shall be a compound which is capable of binding to the main celiac epitope with an affinity or avidity high enough to replace, at least partly, patient celiac antibodies or vice versa. The test compound should bind the main celiac epitope with a binding affinity or avidity sufficiently high to compete with patient autoantibodies, preferably with a binding affinity or avidity higher than that of the patient antibodies. A skilled person will understand that even if the binding affinity or avidity of the test compound is comparable with or lower than that of the patient
autoantibodies, a sufficient degree of replacement can be achieved by using a higher concentration of
the test compound.

In an alternative embodiment displacement of the test compound by celiac patient antibodies is detected.

5 Setting the concentration to achieve an appropriate replacement of the autoantibodies by the test
compound is well within the skills of a person skilled in the art.

A preferred test compound is an antibody or antibody fragment highly specific for the main celiac
epitope. A particularly preferred test compound is a monoclonal antibody raised against said epitope.

A preferred example is Mab885 of Phadia deposited on March 25, 2010 according to the Budapest
Treaty by Phadia AB (P.O.Box 6460 751 37 UPPSALA, Sweden, Visiting address: Rapsgatan 7P 754
50 Uppsala) under Accession number ............... at DSMZ - Deutsche Sammlung von
Miroorganismen un Zellkulturen GmbH, Inhoffenstr. 7 B, D-38124 Braunschweig, Germany as
indicated in the appropriate international form.

In the preferred embodiment the test compound is a test antibody or fragment, variant, derivative or
analogue thereof known to be capable of binding to the main celiac epitope of a protein belonging to
the transglutaminase family, said protein being an autoantigen in celiac disease, preferably TG2, TG3
or TG6, more preferably TG2 or TG6, highly preferably TG2.

In the preferred embodiment the test compound is a fragment, variant, derivative or analogue of the
Mab885 comprising a binding region having the amino acid sequence of a variable region of Mab885
or an amino acid sequence having at least 60%, 70%, 80%, 90% 95%, 98% or 99% sequence identity
therewith, said a fragment, variant, derivative or analogue being capable of binding to the main celiac
epitope of a protein belonging to the transglutaminase family.

Expediently, the biological sample taken from said subject is a sample of a tissue or body part, either
in native or processed state, wherein celiac autoantibodies can reside. In a preferred embodiment of
the invention the biological sample taken from said subject is a body fluid sample, e.g. in saliva,
duodenal juice, stool or a blood sample, or a processed sample thereof, e.g. a serum sample or a
sample containing appropriate inhibitors.

In further embodiments the biological sample is a solid tissue sample wherein celiac autoantibodies
are deposited e.g. preferably a sample taken from small intestine, jejunum, placenta or liver
[Corponay-Szabo, L.R. et al. Gut 53, 641-648 (2004)]. The sample can be taken from the skin wherein
the presence of anti TG3 antibodies are reported.

The advantage of using a body fluid sample is that patient autoantibodies are present in the sample in
a directly applicable form and no isolation thereof is necessary. Preferably, from solid tissue samples
patient autoantibodies are to be obtained by at least partial isolation or elution of the autoantibodies
from the sample as described in Salmi TT et al, Gut, 2006;55(12):1746-53.].
The invention also provides for the use of a test compound as disclosed herein, said compound specifically binding to the main celiac epitope, for diagnosis of celiac disease or in a method for diagnosis of celiac disease in a displacement assay.

In the use of the present invention patient antibodies and the test compound of the invention both bind to the main celiac epitope and thereby compete for the binding site. Assay conditions can be set either in such way that celiac patient autoantibodies replace the test compound or alternatively in such a way that the test compound replaces the celiac patient autoantibodies. According to a further aspect of the invention a further diagnostic kit for the diagnosis of a gluten-induced autoimmune disease in a subject is provided, said kit comprising

a) a test compound known to be capable of binding to the main celiac epitope of a protein belonging to the transglutaminase family, said protein being an autoantigen in celiac disease;

b) a medium carrying instructions for providing and using a reference protein belonging to the transglutaminase family, said protein having an integral main celiac epitope;

c) a reference protein belonging to the transglutaminase family, said protein having an integral main celiac epitope and

d) means for taking a biological sample from a subject, said sample containing autoantibodies of said subject and/or means for isolating autoantibodies from said sample, and/or

d) means for assessing the level of binding and/or kinetics of the autoantibodies to the proteins.

Preferably the test compound is a test antibody or fragment, variant or analogue thereof known to be capable of binding to the main celiac epitope of a protein belonging to the transglutaminase family, said protein being an autoantigen in celiac disease. More preferably the test antibody is a celiac patient derived antibody or a fragment, a variant or an analogue thereof having the binding site and/or the recognition pattern typical of a patient derived antibody.

Preferably, the test antibody is a monoclonal antibody or fragment, variant or analogue thereof having a binding site of the same binding properties. In a highly preferred embodiment the monoclonal antibody is an antibody capable of binding to the surface part of the first one to four amino acids of the first alpha helix of the core domain. Preferably, the antibody is the Mab 885 monoclonal antibody.

In a further aspect the invention relates to a use of a test protein in a method for diagnosis of a gluten induced autoimmune disease,

said test protein belonging to the transglutaminase family, in which the side chain and/or spatial position of at least one surface amino acid residue contributing to the main celiac epitope is altered as compared to the reference protein in which the main celiac epitope is integral,

wherein the said at least one surface amino acid residue of the main celiac epitope comprises or is selected from

a surface amino acid residue of the first alpha helix of the core domain, preferably a surface amino acid residue selected from the first four, three, or two
amino acid residues of said alpha helix, more preferably the first amino acid residue of said alpha helix, and/or a surface amino acid residue of the first alpha helix of the beta-sandwich domain and a surface amino acid residue of the conserved HisHisThr motif of the beta sandwich domain, preferably a surface amino acid residue selected from the last six, five, four or three amino acid residues of said first alpha helix of the beta-sandwich domain and amino acid residues of the HisHisThr motif, more preferably the sixth amino acid residue of said helix and/or the first amino acid of said HisHisThr motif.

and wherein the core domain has a folded three dimensional structure, preferably a native folded three dimensional structure.

Preferably, in the use of the present invention, the test protein and the reference protein is any test protein and reference protein, respectively, as defined herein.

Preferably, in the use of the present invention the method for diagnosis of celiac disease is any diagnostic method as described herein.

For example, in the use, diagnostic method or test kit according to the invention

the test protein is a mutant of a wild type protein belonging to the transglutaminase family and being an autoantigen in celiac disease, and

the reference protein is wild type protein belonging to the transglutaminase family which is an autoantigen in celiac disease.

Preferably, the reference protein is a TG2, TG3 or TG6 comprising wild type amino-terminal beta-sandwich and core domains, and the test protein is a TG2, TG3 or TG6 comprising mutant beta-sandwich and core domains.

In a further aspect the invention relates to a preparation method for preparing a test protein belonging to the transglutaminase family useful in a diagnostic method for diagnosing celiac disease in a subject, comprising the steps of

i) selecting a reference protein belonging to the transglutaminase family in which the celiac epitope is integral,

ii) designing a mutant variant of said protein wherein the side chain and/or spatial position of at least one surface amino acid residue contributing to the main celiac epitope is altered as compared to the reference protein in which the main celiac epitope is integral,

wherein the said at least one surface amino acid residue of the main celiac epitope comprises or is selected from

a surface amino acid residue of the first alpha helix of the core domain, preferably a surface amino acid residue selected from the first four, three, or two amino acid residues of said alpha helix, more preferably the first amino acid residue of said alpha helix, and/or
a surface amino acid residue of the first alpha helix of the beta-sandwich domain
and a surface amino acid residue of the conserved HisHisThr motif of the beta
sandwich domain, preferably a surface amino acid residue selected from the last
five, four or three amino acid residues of said first alpha helix of the beta-
sandwich domain and amino acid residues of the HisHisThr motif, more
preferably the sixth amino acid residue of said helix and/or the first amino acid
of said HisHisThr motif,

and wherein the core domain has a folded three dimensional structure, preferably a native folded three
dimensional structure

iii) preparing a mutant recombinant nucleic acid coding for a mutant amino acid sequence comprising
said mutation,

iv) expressing said mutant recombinant nucleic acid in a host,

v) obtaining said mutant protein from said host to provide a test protein which, upon contacting it
with a celiac antibody shows reduced binding level (preferably at least 30% lower, more preferably at
least 40% lower, even more preferably at least 50% lower level of binding) as compared to the
corresponding wild type reference protein.

In a further embodiment the invention relates to a preparation method for preparing a reference
protein belonging to the transglutaminase family useful in a diagnostic method for diagnosing celiac
disease in a subject, comprising the steps of

i) selecting a test protein belonging to the transglutaminase family in which the celiac epitope is
impaired, deficient or not present,

ii) designing a mutant variant of said test protein wherein the side chain and/or spatial position of at
least one surface amino acid residue contributing to the main celiac epitope is altered as compared to
the test protein in which the main celiac epitope is impaired to obtain a reference protein in which the
main celiac epitope is integral,

wherein the said at least one surface amino acid residue of the main celiac epitope comprises or is selected from

a surface amino acid residue of the first alpha helix of the core domain,
preferably a surface amino acid residue selected from the first four, three, or two
amino acid residues of said alpha helix, more preferably the first amino acid
residue of said alpha helix, and/or

a surface amino acid residue of the first alpha helix of the beta-sandwich domain
and a surface amino acid residue of the conserved HisHisThr motif of the beta
sandwich domain, preferably a surface amino acid residue selected from the last
five, four or three amino acid residues of said first alpha helix of the beta-
sandwich domain and amino acid residues of the HisHisThr motif, more
preferably the sixth amino acid residue of said helix and/or the first amino acid of said HisHisThr motif, and wherein the core domain has a folded three dimensional structure, preferably a native folded three dimensional structure

i) preparing a mutant recombinant nucleic acid coding for a mutant amino acid sequence comprising said mutation,

iv) expressing said mutant recombinant nucleic acid in a host,

v) obtaining said mutant protein from said host to provide a reference protein which, upon contacting it with a celiac antibody shows increased binding level (preferably at least 30% higher, more preferably at least 40% higher, even more preferably at least 50% or 60% higher level of binding) as compared to the corresponding wild type reference protein.

DEFINITIONS

The fold of a protein or domain structure is understood herein as the spatial (three dimensional) arrangement of its secondary structural elements (including major structural elements, e.g. helices, e.g. alpha-helices, and extended structures, e.g. beta structures, e.g. beta strands, parallel and antiparallel beta sheets, beta barrels, and minor structural elements, e.g. various turns, e.g. beta or gamma turns, loops etc.) relative to each other.

A secondary structural element (SSE) is a unit of secondary structure of a given part or segment of a protein having a defined conformation. SSEs can be assessed, predicted or calculated by various methods (see below). For example, according to an embodiment two or optionally three types of SSEs are used are used: alpha-helix, extended structure and optionally coils or random structures (see below).

The fold of two proteins or two protein domains are considered herein to be analogous or similar if at least the majority of, preferably at least 60%, 70%, 80% or at least 90% of or each of the major SSEs of one of the proteins or domains can be corresponded to those of the other. In case its fold is at least 80%, preferably at least 90% identical they are called essentially identical. This is the case e.g. if to a given secondary structure element in a first protein or domain which is connected or adjacent to given further elements, a corresponding element of the same type can be found in a second protein or domain which is connected or adjacent to elements of the same type as the given further elements in the first protein or domain. The ratio of the corresponding SSEs can be calculated by number of amino acids or by number of the SSE.

The fold of a protein after any event or effect remains essentially intact or is maintained if any of the following criteria are fulfilled:

i) the spatial arrangement of at least the major SSEs (helices, e.g. alpha helices and extended structures, e.g. beta structures, like beta sheets and beta barrels) is maintained and/or if the fold remains analogous or similar or essentially identical,
ii) the ratio of the major structural elements is changed by at most 40% or 30%, preferably by at most 25% or 20%, more preferably by at most 15%, 10% or 5% and the ratio of the random structures is changed by at most 40% or 30%, preferably by at most 25% or 20%, more preferably by at most 15%, 10% or 5%,

5 iii) any activity of the protein remains detectable,

iv) any composite or conformational (non-continuous) epitope of the protein remains immunologically detectable.

It is to be understood that any art methods useful for studying said criteria can be applied in the present invention. The skilled person will know that all these methods have their limitations so the definition above is to be interpreted necessarily in view of these limitations.

A protein or protein domain, including any mutant, variant or homologue thereof has a native fold if its fold is similar or analogous to or essentially identical with a wild type protein. A transglutaminase fold of a protein or domain is understood herein as a fold analogous or similar or essentially identical to the fold of a TG family protein or a respective domain thereof (e.g. domain I, II, III or Fv), preferably wherein said TG family protein or a respective domain thereof (e.g. domain I, II, III or Fv), preferably wherein said TG family protein may be an autoantigen in celiac disease and preferably wherein said TG family protein is a eukaryotic, an animal, a vertebrate or mammalian protein.

A folded three dimensional structure of a protein or domain is understood herein as a structure wherein spatially (three dimensionally) defined structural elements, e.g. SSEs (including major structural elements, e.g. helices, e.g. alpha-helices and extended or beta structures are arranged in a spatially defined albeit flexible tertiary structure which provides a certain extent of thermodynamic stability to the protein or domain under the conditions given. Alternatively, a folded protein or domain has a definite or definable fold. An unfolded protein or domain or a molten globule state is not considered as folded.

A folded three dimensional structure of a protein or domain is native if it has a native fold.

In preferred embodiments, in a folded or native folded three dimensional structure the fold of a protein or domain remains essentially intact or is maintained. As immediately apparent to a skilled person this can be experimentally shown by a number of structure analysis method. Any of these methods can be used to characterize the folded nature of the said protein or domain, with the proviso that the definition of folded nature in this case is limited or bound by the method applied.

As also apparent to a skilled person that if the protein remains active, the protein must be essentially or at least partly folded which is to be considered as folded herein. Furthermore, if composite or conformational (non-continuous) epitopes are present, the protein or domain is also to be considered as folded and this fact can be detected by an appropriate antibody.

A mutant of a given protein or protein domain is a protein or protein domain in the sequence of which one ore more amino acid residues has been deleted, substituted or inserted by genetic engineering or random mutagenesis method as compared to the sequence of the given protein or protein domain.
A **variant** of a given protein or protein domain is a protein or protein domain the sequence of which differs in one or more amino acid residues as compared to the sequence of the given protein or protein domain, wherein the variant may comprise deletions, substitutions or insertions.

A **homologue** of a given protein or protein domain or a protein or domain homologous thereto has a sequence identity of at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95%, and its fold is analogous or similar. Preferably, at least 50%, 60%, 70%, 80%, 90% or 95% of the conserved regions of the given protein family are the same in a protein or domain and its homologue.

A **mutant, variant or homologue** has preferably an analogous or essentially identical fold as the given protein or protein domain, and even more preferably the amino acid sequence identity between said mutant, variant or homologue, independently from each other, and the given protein or protein domain is at least 40%, preferably at least 50%, at least 60% or more preferably at least 70%, at least 80% or at least 90% or 95%. In case of a mutant or variant in a preferred embodiment the mutant or variant differs from the given

**Altering the side chain** of an amino acid residue in a given protein, i.e. in the amino acid sequence or the three dimensional structure may comprise altering the side chain geometry, side chain size, side chain functional group, or side chain charge either by mutagenesis, including site directed or random mutagenesis or by chemical derivatization.

**Side chain geometry** of an amino acid present in a polypeptide or protein molecule can be characterized by any method or model appropriate for molecular geometry calculation. For example, side chain geometry can be understood for example as the shape of a three dimensional surface of the side-chain (enclosing the molecular volume), e.g. the van der Waals surface, or a spatial arrangement of atomic nuclei of a set of atoms, either with or without hydrogen atoms, forming the side chain relative to each other etc. Side chain geometry can be calculated by any suitable mathematical method as well-known to a person skilled in the art.

**Side chain size** of an amino acid present in a polypeptide or protein molecule relates to any geometrical measure related or characteristic to the size of said side chain, e.g. length (expressed e.g. in pm or \( \text{Å} \)), surface area (expressed e.g. in \( \text{pm}^2 \) or \( \text{Å}^2 \)) or molecular volume (expressed e.g. in \( \text{pm}^3 \) or \( \text{Å}^3 \)) of a side-chain of said amino acid.

**Side chain functional group** is understood herein as a chemical functional group present in the side chain of an amino acid present in a polypeptide or protein molecule.

The **spatial position** of an amino acid residue in a polypeptide or protein molecule i.e. in a three dimensional protein structure, e.g. transglutaminase, is understood herein as a set or entirety of the positions of its atoms in the three dimensional structure of the protein, or a set or entirety of its atomic or position coordinates in a coordinate system taken for, defined by or fitted to the given protein three dimensional structure. Thus, **altering the spatial position of an amino acid residue** is to be understood as altering or mutating the protein structure so that these positions or coordinates are altered in a higher degree than those resulted from flexibility or conformational movement of that protein under
the conditions given. For example, the alteration of a spatial position of an amino acid may result from a mutation in the vicinity or neighbourhood of said amino acid resulting a change in the side chain position or conformation or from a mutation effecting also the position of the alpha carbon atom or the peptide backbone related to that amino acid.

In particular, in proteins belonging to the transglutaminase family and having a folded beta-sandwich domain and a folded core domain such an alteration in spatial positions of a surface amino acid residue of the first alpha helix of the core domain, and a surface amino acid residue of the first alpha helix of the beta-sandwich domain may be characterized by a distance between these sets of amino acids i.e. their spatial positions relative to each other.

The expressions transglutaminase superfamily or transglutaminase family of proteins (TG family proteins) are used interchangeably and are understood herein as a class of proteins categorized as EC 2.3.2.13, said proteins having a domain structure comprising an N-terminal beta-sandwich domain, a core domain which is the catalytic domain in these proteins when they have activity, a first beta-barrel domain and a second beta-barrel domain (also called domains I, II, III and IV domain II being the core domain) or a mutant or fragment thereof comprising at least a folded N-terminal beta-sandwich domain and a folded core domain. As a review, see e.g. [Lorand, L., and Graham, R. M., Nat Rev Mol Cell Biol 2003, 4, 140-156]. A protein belonging to the transglutaminase family as defined above is referred herein in short as a TGfamily protein.

Preferably, the core domain has a fold analogous to or essentially identical with the core domain of any of the products of any of the following genes: F13A1, TGM1, TGM2, TGM3, TGM4, TGM5, TGM6, TGM7 or EPB42 as indicated for example on Table 1 and Figure 7 of Grenard et al. [J. Biol. Chem. (2001) vol. 276(35) pages 33056-33078], or any eukaryotic equivalent or homologue thereof provided that it has said core domain with said fold, or any mutant, variant or homologue thereof. Preferably, in said gene products the beta-sandwich domain has a first alpha helix of the beta sandwich domain and said core domain also has the first alpha helix of the core domain as defined below or in the Brief description of the invention.

The first alpha helix of the core domain of a protein belonging to the transglutaminase family is the first alpha helix from the N-terminal of the core domain or domain II (which is the catalytic domain of active members of the transglutaminase family but being also present in the inactive members of the family in a catalytically inactive form). The first alpha helix of the core domain is adjacent to the first alpha helix of the beta-sandwich domain in the native transglutaminase fold. Thus, the first alpha helix of the core domain is an alpha helical SSE corresponding to any of the following alpha helical secondary structure elements (SSE):

an SSE spanning from Glu153 to at least Tyr159 or Val160 in human transglutaminase 2,

an SSE spanning from Glu198 to at least Tyr204 or Val205 in human factor XIII,

an SSE spanning from Glu153 to at least Tyr159 or Val160 in human transglutaminase 1,

an SSE spanning from His148 to at least Tyr154 or Val155 in human TG3,
as indicated e.g. in Lorand and Graham, Nature Reviews 2003, Vol. 4, page 140, figure 3.
The first alpha helix of the beta sandwich domain of a protein belonging to the transglutaminase
family is the first alpha helix from the N-terminal of the N-terminal beta-sandwich domain or domain
t. The first alpha helix of the beta-sandwich domain is adjacent to the first alpha helix of the core
domain in the native transglutaminase fold. Thus, the first alpha helix of the beta sandwich domain is
an alpha helical SSE corresponding to any of the following alpha helical secondary structure elements
(SSE):
an SSE spanning from Ser14 to His21 in human transglutaminase 1,
an SSE spanning from Leu14 to His21 in human transglutaminase 2,
an SSE spanning from Thr12 to His19 in human TG3,
an SSE spanning from Trp57 to His64 in human factor XIII,[see Lorand and Graham, Nature Reviews Volume 4, page 140, 2003].
The first, or the first n amino acid residue(s) of an SSE is(are) the first or the first n amino acid
residue(s) of the SSE from the N-terminal, i.e. the most amino-terminally located amino acid
residue(s) of said SSE wherein n is a positive integer. The last or the last n amino acid residue(s) of
an SSE is(are) the last or the last n amino acid residue(s) of the SSE from the C-terminal, i.e. the most
carboxy-terminally located amino acid residue(s) of said SSE wherein n is a positive integer. The
ascertainment whether an amino acid residue belongs to a given SSE can be based on the three
dimensional structure of the protein or homologous proteins if related by sequence alignment or
homology modelling or by any other appropriate method and/or based on the torsion angle value(s) of
the respective amide plane(s) and/or by any appropriate detection, calculation or prediction method.
The position of an amino acid residue which is in n amino acid distance from a given amino acid in
either C-terminal (carboxy-terminally) or N-terminal (amino-terminal) direction is to be calculated by
counting each of n amino acid residues in the said direction starting from the first amino acid which is
adjacent to the given amino acid in the said direction, wherein n is a positive integer.
A gluten-induced autoimmune disease involves any disease, either in latent or explicit (overt) form,
the symptoms of which can be induced by gluten and during the course of the disease autoantibodies
are elicited in the patient against a transglutaminase (TG), preferably against TG2, TG3 or TG6.
Gluten-induced autoimmune diseases typically include e.g. celiac disease (also known as coeliac
disease, non-tropical sprue or gluten-sensitive enteropathy) which typically develops among
genetically predisposed persons with the human leucocyte antigen (HLA) DQ2 or DQ8 background,
and dermatitis herpetiformis in which, in addition to disorders in the intestine, there are granular IgA
depositions in the skin subpapillary areas.

An epitope is a patch or site on a protein molecule, preferably a part of its molecular surface, to which
an antibody or the binding region of an antibody can bind.
An epitope can be defined e.g. by giving the amino acid residues contributing to this patch or site of
the molecule or the formation of the given molecular surface, or coordinates thereof; by giving its
location on the protein molecule in terms of structural elements contributing to the fold of said protein; or by defining the molecular surface part by mathematical calculation methods.

A mimotope of an epitope is a patch or site on a molecule, preferably a part of its molecular surface, to which the same antibody or the binding region can bind as to said epitope.

Epitopes as molecular surface parts of a molecule can by given and mimotopes can be prepared e.g. as described [Goede, Andean et al.: BMC Bioinformatics 2005, 6:223].

A main celiac epitope is understood as an epitope for which at least one of the following features are true:

a) a part of the molecular surface of a TG family protein to which a disease specific autoantibody of a subject with celiac disease is capable of binding, wherein said autoantibody is capable of binding to said part of the molecular surface whereas autoantibodies of a subject with any other disease having autoantibodies capable of binding to TG2 usually or typically do not bind to said part of the molecular surface; or

a part of the molecular surface of a TG family protein to which or to a part of which Mab 885 is capable of binding, optionally including a surface area within 2, 3, 4, 5, 6, or 7 Å from the area covered by Mab885 when bound, or

a part of the molecular surface of a TG family protein, to which an celiac? autoantibody of a subject with celiac disease is capable of binding as explained above, in a manner that said autoantibody can be displaced in a competitive assay by Mab 885;

b) a part of the molecular surface of a TG family protein which may be an autoantigen in celiac disease, said molecular surface part at least partially formed or contributed by

one or more surface amino acid residue(s) of the first alpha helix of the core domain, preferably a surface amino acid residue selected from the first four, three, or two amino acid residues of said alpha helix, more preferably the first amino acid residue of said alpha helix,

and/or

one or more surface amino acid residue(s) of the first alpha helix of the beta-sandwich domain and the conserved HisHisThr motif of the beta sandwich domain, preferably a surface amino acid residue selected from the last six, five, four or three amino acid residues of said first alpha helix of the beta-sandwich domain and amino acid residues of the HisHisThr motif, more preferably the sixth amino acid residue of said helix and/or the first amino acid of said HisHisThr motif;

c) a part of the molecular surface of a TG family protein which may be an autoantigen in celiac disease, overlapping with or comprising a surface part within a sphere having a 6, 7 Å radii calculated from any atom of the Glu153 and/or within a sphere having a 6, 7 Å radii calculated from any atom of the Arg9 according to the amino acid numbering of the full length human TG2, or an amino acid residue corresponding/equivalent thereto in, respectively, in an animal (preferably vertebrate, more preferably mammalian) TG2 based on multiple sequence alignment;
d) a part of the molecular surface of a TG family protein which may be an autoantigen in celiac disease, at least partially formed by amino acid residue corresponding/equivalent to Glu153 and/or Arg19 numbered according to the amino acid numbering of the full length human TG2 based on multiple sequence alignment;

e) a patch of a molecular surface of a molecule, e.g. a part of the molecular surface in a protein, preferably a TG family protein, which is capable of binding a surface recognition molecule, preferably an antibody, wherein said surface recognition molecule is capable of binding to a main celiac epitope as defined in a) to d), wherein preferably said TG family protein is

- a eucaryotic protein, preferably an animal protein, more preferably a vertebrate or mammalian or human protein,
- a TG2 and/or TG6 protein,
- an engineered TG family protein, preferably wherein a main celiac epitope is created by mutation or wherein a main celiac epitope is impaired by mutation.

The main celiac epitope can be considered as integral if it is intact, i.e. has a molecular surface essentially identical with the molecular surface of a wild type protein of the TG family which is autoantigen in celiac disease, and/or a celiac antibody known to be capable of binding the main celiac epitope, e.g. to that epitope of TG2, is capable of detectably binding to it, and/or the side chain geometry, side chain size, side chain functional group, charge and spatial position of at least the first N-terminal surface exposed amino acid residue of the N-terminal alpha helix of the core domain, as defined below, are essentially identical with or immunologically indistinguishable from the same values for a corresponding wild type protein of the transglutaminase family (TG family protein) which may be an autoantigen in celiac disease, and/or it is altered as compared to the test protein in which the main celiac epitope is impaired to obtain a reference protein in which the main celiac epitope is integral or recognizable by celiac antibodies. A main celiac epitope is impaired or deficient if in comparison with the main celiac epitope of a wild type transglutaminase, which is an autoantigen in celiac disease, it provides a different molecular surface and therefore a celiac antibody can be found which bind to the impaired main celiac epitope with a lower affinity or avidity, or it can not bind.

In an embodiment, binding of a celiac antibody to a test protein in which the celiac epitope is different from an intact celiac epitope is impaired if the binding affinity and/or avidity and/or rate of the binding reaction is reduced as compared to a reference protein, preferably the average or mean binding level is reduced by at least a predetermined ratio, preferably by at least 30%, more preferably by at least 40%, even more preferably by at least 50%).

A celiac antibody is an antibody capable of specifically recognizing and binding to the main celiac epitope.
A celiac autoantibody is a celiac antibody which is an autoantibody of a subject in celiac disease wherein said autoantibody is capable of binding to a wild type TG family protein, wherein said protein is an autoantigen in celiac disease, preferably TG2 and/or TG6 and optionally TG3, more preferably TG2, and/or it is capable of binding at the main celiac epitope as defined above.

Endomysial antibody is a celiac antibody capable of binding to the connective tissue structure surrounding smooth muscle cells in human or primate esophagus or of other tissue sections containing smooth muscle cells and which antibody is detected on frozen tissue sections by indirect immunofluorescent method.

The molecular surface of a protein molecule, e.g. a TG family protein, is the surface area which describes the three dimensional surface of said protein molecule which is accessible to other chemical entities, e.g. to the solvent or other molecules, e.g. ions or protein molecules, e.g. antibodies. For the purposes of the present description the meaning of molecular surface also includes the "accessible surface area" or "Lee-Richards molecular surface", the solvent-excluded surface or Connolly surface or the Van der Waals surface. Thus the molecular surface can be calculated by any algorithm suitable for calculation of such three dimensional surfaces irrespective of variations between calculation methods as so far as the model used for calculation provides a scientifically correct estimation of the surface.

Binding affinity, as used herein is a thermodynamic expression of the strength of interaction between an antigen binding site and an antigenic determinant (and thus of the stereochemical compatibility between them). In a preferred meaning, the term is applied to interactions among antigenic determinants and antibody binding sites. Binding affinity can be characterized by a quantity or measure calculated from or correlating to the dissociation constant or a function thereof, e.g. the reciprocal of the dissociation constant 1/K_d (association constant) or the negative logarithm of the dissociation constant or a function thereof, such as −log_{10}[K_d/NA * (mol/l)^n] which is also indicated as pK_d. For example, binding affinity can be characterized by the quantity or ratio of the bound molecules, e.g. ligands or antibodies, in or after an equilibrium state. Typically, owing to the heterogeneity of affinities in a population of antibody molecules of a given specificity, binding affinity (and e.g. the association constant or the pK_d) actually represents a kind of average value.

Avidity, as used herein in an aspect relates to the combined strength of affinities in a protein-ligand complex. In an other aspect, avidity describes the binding intensity of multiple bond interactions between proteins. Preferably, avidity refers to the strength of antibody-antigen binding. Avidity is meant herein as a term including affinity but more broadly as avidity is also applicable to describe strength of a multiple bond. For instance, a single binding site of IgM may have low affinity but still IgM has high avidity due to its 10 weak binding sites contrary to the two strong binding sites of IgG.

An inhibitor of the binding of a celiac antibody to a celiac epitope is a compound capable of binding to either a TG2 (transglutaminase) or a celiac antibody and upon binding of which the detectable level or affinity of binding is reduced as compared to the binding in absence of said inhibitor.
BRIEF DESCRIPTION OF THE FIGURES


(B) Transglutaminase (TGase) and GTPase activities of the mutant proteins as percentages of respective values of wild type TG2. Values are presented as means from two separate measurements done in triplicate.

2. Fibronectin binding of TG2 mutants. Proteins directly coated to the plate (aTG-ELISA) or added to fibronectin were measured by monoclonal anti-TG2 antibody TG10 recognizing the core domain (II). Results showed equal antigen amounts on the plate in the assays and relative to each other. These amounts were used in further measurements with celiac samples. Values are presented as means from two separate experiments done in duplicate. For the abbreviation of proteins see Figure 1.

3. Binding of serum IgA antibodies from celiac patients (n=14) to Site 4 mutants (A) and domain mutant TG2 proteins (B) in ELISA performed with direct coating of antigens to the plate. Results are shown as percentages of the binding relative to wild type TG2. S4, Site 4 mutant, S4.1/4/5, D151N-E154Q-E155Q combined mutant.

4. Binding of serum IgA antibodies from celiac children presenting with severe malabsorption (upper panel) and adult patients with malabsorption (lower panel) to mutant TG2 proteins. Anti-TG2 ELISA performed by direct coating of antigens to the plate. Binding results are shown as percentages of amounts bound to wild type TG2 in comparison to anti-TG2 monoclonal antibody TG10. All serum samples were examined in duplicates. Dashes indicate medians. For the abbreviations of mutants see Figure 1.

5. (A) Structure of the putative celiac epitope in TG2 and Factor XIII. (B) Binding of serum IgA antibodies of celiac children (n=20) and adults (n=17) to wild type TG2 and TG2 mutants mimicking the surface of Factor XIII. K, E154K, RKM, R19S-E154K-M659S triple mutant.

6. Binding of serum IgA antibodies from celiac children and adults to fibronectin-bound TG2 mutants in ELISA. Results are shown as percentages of amounts bound to wild type TG2 in comparison to anti-TG2 monoclonal antibody TG10.

7. Structure of the celiac epitope of TG2 in closed and open form of the protein.

8. Disease specificity of the celiac epitope targeting. Binding of anti-TG2 antibodies from celiac disease patients and from patients with other autoimmune diseases (n=11) to wild type and mutant TG2 proteins.

9. Celiac disease antibodies from different patients recognize the same epitope in competition assays. (A) Serum samples from 56 IgA deficient celiac patients containing IgG class anti-TG2 antibodies
compete with the same celiac IgA in ELISA using wild type TG2 and the competition is proportional to their serum concentrations (A2). Three of the patients also have IgM class anti-TG2. Serum samples from non-celiac IgA deficient (n=23) and IgA competent subjects (n=22) do not show interference (A1). (B) Purified IgG celiac antibodies reacting with the N-terminal R19S mutant (GI) or not reacting with the R19S mutant (G5) but both equally non-reactive to R19S-E153S composite (RE) mutants compete in the same dose-dependent manner with purified celiac IgA not reacting to R19S, whereas total IgG fraction (K4) prepared from non-celiac subject does not show interference. IgA and IgG antibodies bound to ELISA plate were separately recognized.

10. Correlation of bindings of celiac antibodies to Site 4 and Site 5 mutants compared to wild type TG2

11. Stability of the celiac epitope targeting in celiac patients undergoing diagnostic gluten challenge after a period of diet and seronegativity (A-B) and in a celiac patient followed up for 5 years without diet (C).

12. Tissue deposited and passively transferred pathogenic anti-TG2 antibodies have same epitope specificity as the antibodies in the serum of celiac patients. (A) Maternal anti-TG2 IgA antibodies deposited on the surface of chorionic villous structures of the infant and in the maternal parts of the placenta in a celiac woman with active disease. Passively transferred maternal IgG deposited in umbilical cord tissues of the newborn as endomysial antibodies. (B) Antibodies eluted from these tissues show similar epitope specificity as serum antibodies using TG2 mutant proteins. (C) Human umbilical cord vein endothelial cells (HUVEC) from a newborn with maternal celiac antibodies show abnormal shape, spreading and decreased cell lengths compared to HUVECS prepared from a newborn of a celiac mother on diet and without anti-TG2 antibodies.

13. Measurement of binding kinetics of purified celiac IgA to the same amounts of wild type and Site 4 mutant TG2 using plasmon surface resonance. Decreased binding and faster dissociation are observed.

14. Similar epitope specificity of serum antibodies in subjects with latent (CDL, n=11) and overt (CD, n=11) celiac disease.

15. Frozen small bowel section from a non-celiac subject incubated with serum antibodies from celiac patients and monoclonal mouse antibodies 885 (A) and CUB7402 (B). Bound human IgA was recognized with fluorescent isothiocyanate-conjugated green fluorescent secondary antibodies and mouse antibodies were labelled with rhodamine-conjugated red fluorescent secondary antibodies. Under these conditions, 885 was not able to bind to the tissue along the endomysial structures where patient antibodies bound. CUB bound to endomysial structures along the binding of patient IgA resulting in a merged yellow color.
DETAILED DESCRIPTION OF THE INVENTION

The present inventors studied human TG2 applying a complex structural, biochemical and immunological approach.

The monomeric human TG2 consists of 687 amino acids with a molecular weight 76 kDa and is composed of four structural domains: N-terminal beta-sandwich domain with a fibronectin-binding region, catalytic core domain with the catalytic triad, and two C-terminal beta-barrel domains. The GDP-bound form of TG2 has been crystallized a few years ago, which is the "closed" conformation of the enzyme [Liu S et al, Proc Natl Acad Sci U S A. 2002;99(5):2743-7.]. The inventors started the localization of the potential binding sites of celiac antibodies with this model. In this conformation the GDP is bound in a cleft between the catalytic core and the first β-barrel, and the catalytic triad, which is responsible for the transamidation activity, is hidden inside the molecule and it is inhibited by two loops, so the enzyme can not operate as a transglutaminase. Recently, the enzyme's "open" form has been crystallized in a complex with a gluten peptide-derived inhibitor [Pinkas DM et al, PLoS Biol. 2007;5(12):e327.]. In this case the C-terminal β-barrel domains are displaced by 120 Å compared to the GDP-bound form and the active site residues become accessible to the substrates. This activated form of TG2 is "frozen" in this model with the active site inhibitor bound to the catalytic amino acids. Despite this finding, a Ca²⁺-bound form of TG2, without substrate or inhibitor, is still unknown. It is still a question, which form of TG2 appears extracellularly, where it is recognizable for the celiac autoantibodies. After a series of protein engineering experiment the present inventors found that two anchor sites in the TG2 structure are essential in forming an epitope for patient autoantibodies in gluten induced autoimmune diseases, preferably in celiac disease. It was found that neither the substrate binding site nor Ca²⁺ ions form part of the celiac epitope, however, Ca²⁺ binding site 4 overlaps therewith. The epitope is a conformational one but as the two anchor sites are located on the N-terminal beta-sandwich domain and the core domain, binding of the majority of patient autoantibodies do not block the open-closed conformational transition of the enzyme.

Surprisingly, it has also been found by the inventors that this surface patch of the molecule can distinguish between autoantibodies from celiac patients or patients with a gluten induced autoimmune disease and those from other autoimmune diseases. Thereby a diagnosis highly selective for these diseases can be provided.

As immediately obvious for a skilled person an epitope in such a complex disease can vary from patient to patient or depending on the disease manifestation type or progress of the disease. In light of this general knowledge, it is significant that the composite epitope region found by the inventors is highly characteristic i.e. selective for celiac autoimmunity and that according to measurements with serum samples from celiac adults, only insignificant spreading of the disease epitope occurs in later years.

Evidence is also provided herein that the epitope region found herein is already present in the early preclinical stage of the disease and has also predictive value for diagnosis of celiac disease in later life. However, it is to be realized that the exact celiac epitope may spread to further amino acids as the
disease proceeds and this may be considered in certain embodiments of the tests of the invention. For example, without a limitation, the following amino acids in TG2 or amino acids corresponding thereto may be mutated in order to further reduce autoantibody binding: Arg 19, His22, Asp 151, Glu 153, Glu 154, Arg156, Glu157, Leul 61, Val431, Arg433, Glu435, Met659, Leu661 numbered according to the amino acid numbering of the full length human TG2. Corresponding amino acids may be found based on multiple sequence alignment.

It is obvious that the exact area of the patch of molecular surface considered as an epitope may vary as mentioned above. Thus, it should be understood that amino acid replacements or other mutations outside the most central region of the epitope may effect antibody binding. Such regions, without completeness, can be found in certain regions on amino-terminal beta-barrel domain and on the core domain, e.g. in Ca\textsuperscript{2+} binding site 5. The skilled person, upon designing transglutaminase mutants for use in the present invention, will be able to consider these variations and optimise mutants for a given purpose based on the teaching provided herein.

The inventors at the first time identified, as a conformational epitope, the main celiac epitope sought but not found in the prior art. Several overlapping variants of the main celiac epitope may exists. The fact whether a given antibody binds to the celiac epitope or not can be clearly decided based on the teaching provided herein. The present inventors identified essential parts of the epitope, which provides the skilled person with the key to design diagnostic methods, mutant transglutaminases useful therein, diagnostic kits for that purpose.

Diagnostic Methods and Kits
Based on the teaching provided herein the skilled person will recognize that according to an embodiment of the invention the diagnostic method has two important features. On the one hand, a test TG family protein is to be provided in which the celiac epitope is impaired or deficient or lacking so that celiac autoantibodies can not bind to it or bind only at a reduced level. On the other hand, other epitopes are preferably present to ensure selectiveness of the method, therefore, at least the beta-sandwich domain and the core domain have a folded structure. Preferably any or both of the beta-barrel domains are folded as well. Thus, in the present diagnostic method a positive result very reliably indicates that the patient suffers from celiac disease.

The folded character of a domain can be characterized by several means.

A convenient way is to check activity. Thus, in a preferred embodiment a mutant TG2 has one or more of the following detectable functional activities: transglutaminase enzyme activity, a GTPase activity and/or fibronectin binding. If transglutaminase activity is decreased due to the mutation it may be due to the fact that the mutated site is involved in Ca\textsuperscript{2+} binding.

The fact that a non-celiac conformational antibody is capable of binding to the protein is also indicative of its folded character.

As immediately apparent to a skilled person correct protein folding can be experimentally shown by a number of structure analysis method, for example by CD (circular dichroism) spectroscopy, FT-IR
(fourier-transformed infrared spectroscopy), DSC (differential scanning) microcalorimetry, NMR (nuclear magnetic resonance) spectroscopy, DPI (dual polarisation interferometry), atomic force microscope etc or predicted by the method of Chou-Fasman and GOR methods, neural network models or nearest-neighbor methods, as reviewed e.g. in [Simossis VA, and Heringa J, "Integrating protein secondary structure prediction and multiple sequence alignment", Curr Protein Pept Sci. 2004 5(4) 249-66; Rost B, "Review: protein secondary structure prediction continues to rise" J Struct Biol. 2001 134(2-3) 204-18].

These methods can be used either as a fingerprint of said fold or for detection of any difference between folds of different proteins or domains, e.g. a wild type or a mutant thereof, or between folds of the same protein in different states.

In a real life diagnostic method a reference protein is to be provided which comprises an integral celiac epitope or, if a wild type transglutaminase is used, e.g. a TG2 or TG6, or in certain embodiments TG3, intact celiac epitope. It is to be noted that the molecular surface of the epitope region in TG6 is highly similar to that of TG2, thus, if desired, these epitope regions can be easily converted into each other by site directed mutagenesis. Moreover, provided that autoantibodies against TG6 in a variant of the disease are different from those against TG2, the epitope of TG6 is to be applied for diagnosis of that particular variant of disease. This is also true for further transglutaminases autoantigenic in a gluten induced autoimmune disease.

In a method differential binding of autoantibodies is to be shown. For example, if a value, preferably an average or mean value correlating to the binding affinity and/or avidity and/or rate of the binding reaction is reduced in the test protein as compared to a reference protein, preferably said value is reduced by at least a predetermined ratio, preferably by at least 20%, 30%, more preferably by at least 40%, even more preferably by at least 50% or 60% or 70% or 80%, or the corresponding remaining value is not more than 80%, 70%, more preferably not more than 60%, even more preferably not more than 50%, or 40% or 30% or 20%, respectively, this result is considered as an indication of the presence of the disease. It is also preferred if the mean binding level of the autoantibodies to the reference protein exceeds a predetermined threshold value. This threshold value can be determined by simply carrying out preliminary measurements with a reference transglutaminase using either a standard celiac sample or a sample obtained from a patient known to have celiac disease. A predetermined threshold value need not to be numerically defined in each cases, it may be sufficient if binding is safely detectable. As disclosed herein binding is usually given as a relative value in comparison with the binding of autoantibodies known to have binding properties to a transglutaminase with an integral celiac epitope.

Both the test protein and the reference protein may be a wild type protein or can be prepared by protein engineering from an appropriate scaffold which is sufficiently similar or homologous to a protein which is an autoantigen in celiac disease. For example, without completeness, as such a "scaffold" human or animal TGI, TG3, TG4, TG5, TG7, factor XIII, or EBP42 [see e.g. Lorand, L.,

So as to design an appropriate mutant in a transglutaminase or transglutaminase derived protein it is advisable to identify key amino acid residues. For that purpose, amino acid sequence alignment can be performed as well known in the art and includes means well known in the art, e.g. by method reviewed by Shyu et al. [Shyu et al. Genetic Programming and Evolvable Machines 2004 June; 5(2): 121-144] or by methods available e.g. on the home page of the European Bioinformatics Institute, e.g. ClustalW2, MAFFT, MUSCLE, T-Coffee, etc. and/or by using the Genetic Data Environment (GDE) software package [Smith SW et al. Comput Appl Biosci. 1994 Dec;10(6):671-5.].


Both the test protein and a reference protein can be a protein fragment provided that it meets the requirements defined herein. In a preferred embodiment, nevertheless, the test and reference proteins also comprise a folded N-terminal beta-barrel domain.

It will also be understood that any protein, which is an appropriate scaffold, as explained above, can be used in the present invention. Thus, the protein belonging to the transglutaminase family can be a eukaryotic, more preferably an animal, even more preferably a vertebrate, optionally amphibian, reptilian, fish, avian or highly preferably a mammalian or human protein.

In certain preferred embodiment of the invention the test proteins of the invention are different from mutant proteins of the art, preferably mutants as specifically described in any of the following publications [Sblattero et al. Eur J Biochem. 2002 Nov;269(21):5 175-81, Nakachi K et al. J Autoimmun. 2004 Feb;22(1):53-63. Seissler et al. Clin Exp Immunol. 2001 Aug;125(2):2 16-21.]. Typically these mutants are deletion mutants which, provided that they are impaired or deficient in a celiac epitope, do not comprise both a folded beta-sandwich and core domains. In case the opposite would be true and certain methodology would show in these epitope deficient proteins a folded structure for at least these domains, the mutants are preferably excluded from the claimed scope.

In a certain specific embodiment, if appropriate, the TG2 E153S (E), R19S (R), M659S (M) triple mutant and the R19S (R), M659S (M) double mutant, wherein preferably the sequence comprising no further mutation, is excluded from the claimed scope. In a further specific embodiment, if appropriate,
mutants comprising a TG2 Met659 mutation or an M659S mutation, wherein preferably the sequence comprising no further mutation, preferably are excluded. In a further embodiment mutant transglutaminases disclosed in Kiraly et al. FEBS J. 2009 Dec;276(23):7083-96] are excluded from the scope of the present invention, In an embodiment Met659 is unmutated in TG2. In a further embodiment every mutant disclosed in one or all or any combination of the above publications are excluded from the scope of the present invention. In a further embodiment the diagnostic use of any or all or any combination of the above prior art mutants are excluded from the scope of the present invention.

It is well within the skills of a person skilled in the art to detect autoantibody binding. It is immediately apparent for a skilled person that both kinetic methods as well as method characterizing binding affinity or avidity are applicable. For example, any of the methods below, without any limitation, are applicable:

an immunoassay, e.g. ELISA, RIA, lateral flow, immunoprecipitation
a binding assay, e.g. Biacore, fluorescence quenching.

a spectrophotometric method, e.g. FT-IR, circular dichroism, NMR,
a physico-chemical method, e.g. calorimetry, ultracentrifugation etc.

In a preferred embodiment the diagnostic method is carried out in the form of an immunoassay, like RIA or DELPHIA or preferably in an immunosorbent assay, like ELISA.

In a preferred embodiment of the diagnostic method of the invention the transglutaminase is fibronectin bound. Fibronectin binding provides a preliminary orientation of the transglutaminase protein thereby exposing its celiac epitope to antibodies. Thereby, the diagnostic test becomes more sensitive.

In certain embodiments, however, the object is to provide an assay which selectively differentiates among patient celiac autoantibodies and other antibodies which are not celiac antibodies, i.e. are not typical of celiac disease. In such cases it may be advisable to omit fibronectin to allow access to the whole surface of both the test protein and the reference protein. In this case preferably a non-celiac autoantibody of the celiac patient will bind both to the test protein and the reference protein with nearly equal binding affinity. In a variant of this embodiment the assay is carried out in liquid phase. In both methods the presentation of conformational epitopes are believed to be better to some extent.

In a preferred variant IgA or IgG are measured as autoantibodies, provided that the patients are not deficient in any of these types.

The invention also relates to kits for performing the diagnostic methods as outlined above. These kits may comprise the parts as mentioned above or as useful in carrying out the methods outlined above. A kit necessarily comprises a test protein and at least instructions for obtaining a reference protein or the reference protein as well.

Preferably, the kit comprises a human TG family protein and means for detection of antibody binding thereto. Thus, the kit is advisably an immunological kit, and means for detection include the use of
direct markers and/or secondary markers. Direct markers can be fluorescent markers or radioactive markers, secondary markers can be secondary antibodies, optionally conjugated, e.g. marked or enzymel linked antibodies. Thus the kit may work on ELISA, EMA, DELFIA, RIA etc. principles.

In a preferred embodiment of the invention the test protein of the present invention is added to an assay kit useful as a Tissue Transglutaminase Antibody Assay useful in the detection of celiac disease. Such assay kits, so called second-generation human tissue transglutaminase antibody assays are reviewed by van Meensel et al [Clinical Chemistry 50:11 2125-2135 (2004)]. The assay can be performed essentially as described with the exception of assessing binding of patient autoantibodies also to the test protein and a difference in binding is evaluated in comparison with a TG family protein with an integral main celiac epitope mentioned as a reference protein herein.

It will be understood by a skilled person that taking a calibration curve indicating the signal, e.g. OD in ELISA, in the function of antibody level is preferred to using the signal values themselves, as disclosed by van Meensel et al, above. For example, calibration curves can be taken by using an appropriately diluted antibody with known binding properties, like a TG100 antibody.

Diagnostic Methods, Uses and Kits based on Antibody Displacement

In these embodiments, as defined in the brief description of the invention, if a patient autoantibody is displaced by a preselected test compound specifically capable of binding to the main celiac epitope, than this is indicative of the fact that said patient autoantibody is a celiac autoantibody and therefore of the disease itself, i.e. that said patient suffers from or is susceptible for a gluten induced autoimmune disease or preferably celiac disease.

Preferably, the test compound is a test antibody or fragment, variant or analogue thereof known to be capable of binding to the main celiac epitope of a protein belonging to the transglutaminase family, said protein being an autoantigen in celiac disease, preferably selected from TG2, TG3 or TG6. In principle any antibody binding to the celiac epitope with a sufficient binding affinity or avidity is applicable in this embodiment. Such an antibody can be e.g. an isolated patient autoantibody as in Example 7.

More preferably monoclonal antibodies are prepared against the celiac epitope. Such antibodies can be prepared by known techniques, e.g. the hybridoma method. Techniques for the hybridoma method are well known and are disclosed e.g. in Kontermann, Roland; Dübäl, Stefan (Eds.) Antibody Engineering Series: Springer Lab Manuals 2001, XII, 792 p. ISBN: 978-3-540-41354-7; Gary C. Howard, Matthew R. Kaser (Eds) Making and Using Antibodies: A Practical Handbook, CRC Press, 2006, ISBN: 9780849335280. Moreover, preparation of monoclonal antibody producing hybridomas can be ordered from a number of companies like GL Biochem Ltd. (Shanghai, CH), LC Sciences (Houston, TX, USA) or LAMPIRE Biological Laboratories (Pipersville, PA, USA), FusionAntibodies (Pembroke Loop Rd. BTI 7 OQI, Northern Ireland).

In the present invention antibody selection involves selection for the epitope. In one embodiment, this can be done by selecting clones which secrete antibodies that bind to an intact TG2 or other protein of
the transglutaminase protein but do not bind to a mutant which has an impaired celiac epitope. Alternatively, clones are selected which secrete antibodies which can be displaced by an antibody known to be capable of binding to the celiac epitope as disclosed herein. This can be done by routine immunological testing, e.g. by ELISA as described in the Examples.

Once monoclonal antibodies are obtained in the form of hybridomas they can be sequenced easily. Sequencing services can be ordered e.g. from FusionAntibodies (Pembroke Loop Rd. BT1 7 OQL Northern Ireland) based on cDNA sequence.

In an alternative approach the antibody sequence can be obtained by amino acid sequencing, e.g. by the Edman method. This, while sometimes cumbersome, is a routine method and such service can be ordered from various firms e.g. from Proteome Factory AG Magnusstr. 11D-12489 Berlin Germany. Alternatively, a relatively recent method, shotgun sequencing can be applied [Nuno Bandeira et al. Nature Biotechnology, December 2008, v26, nl2, pp1336-1338].

Usually it may be sufficient to sequence only the variable domains or variable domains plus leader sequence. In certain cases full antibody sequence can be obtained. Based on the sequence the gene of the antibody or its fragments can be cloned and tailored to the given use, e.g. can be humanized [e.g. a HAMA (human anti-mouse antibody) can be prepared; a service also offered by FusionAntibodies], binding affinity can be increased by directed evolution methods etc.

Antibody fragments, like single chain antibody fragments (scFv) and Fab fragments carrying the variable region can be obtained and have several advantages due to their size and reliability. Fab fragments differ from scFv's in that as well as containing variable domains, constant regions and are a dimer linked via two cysteine residues.

Fab's and scFv's offer several advantages over monoclonal antibodies as carriers and lower sensitivity due to their small size and a lower negative response by the human immune system. These fragments can be prepared both from a synthetic DNA or from a monoclonal cell line. Such fragments can even be prepared when only the sequence of the variable region is known.

The skilled person may use the phage display method disclosed e.g. in [Marzari, R. et al. J. Immunol. 166, 4170-4176 (2001)], by which scFv's can be prepared, too. Examplary methods for the preparation of diagnostic antibodies based on the phage display method are described in the Examples.

Based on the teaching provided herein it is well within the skills of a person skilled in the art to identify further test compounds.

It is to be understood herein that instead of antibodies and antibody fragments further recognition molecules, e.g. other protein-based receptors of the art can be used. For example, applying the principles observed with IgG domains, successful attempts have been made to construct binding sites specific to a given target molecule on proteins that originally have no receptor characteristics [Xu, L. et al. Chemistry & Biology 2002, 9, 933-942; Skerra, A. Rev. Mol. Biotech. 2001, 74, 257-275; Nygren P. and Uhlen, M. Curr. Op. Struct. Biol. 1997, 7, 463-469]. Appropriate scaffolds are e.g.
fibronectin type 3 and the lipocalin protein and formation of binding site can be achieved by a
directed evolution technique combined e.g. by a display methodology. Surface of these molecules can
be designed by the methods mentioned above.

Preferably, the test compound should be bound more strongly and/or it should be used in a high
centrailion to displace the autoantibodies.

In a preferred embodiment the binding of the test compound is detected. Binding can be detected by a
labelled compound specifically binding to the test compound. Alternatively, the test compound itself
can be labelled. In such case for example a signal without patient sample or antibodies can be defined
as 0% inhibition (maximum binding of the test compound - no disease) and signal with blank as a
100% inhibition (corresponding to full replacement of the test compound by patient antibodies).

Theoretically it might happen that if the test compound is very strongly bound disease is not
recognized. However, binding levels can be influenced by concentration as well known in the art and
thus it is within the skills of a skilled person to set the assay conditions such as shown in Example 7.

In a preferred embodiment the bound antibodies are detected. In this case e.g. a conjugated secondary
antibody can be used to detect antibody binding. The secondary antibody shall be specific to the test
antibody and should not recognize patient antibodies. In a preferred embodiment the antibody type is
different (e.g. IgA antibody deficient patients are diagnosed by an IgA antibody). Alternatively, the
test antibody carries a specific sequence e.g. it is a non-human antibody. In case of engineered
antibodies or antibody fragments (e.g. scFv or Fab) it is easy to add such sequences which provide
specific recognition sites for secondary antibodies by genetic engineering (Kontermann et al., 2001,
Howard and Kaser, 2006, see above). As known in the art, further to secondary antibodies, antibody
fragments and derivatives other recognition molecules can be used, as well.

In an alternative method the binding of patient antibodies can by detected by a molecule specifically
recognizing such antibodies, e.g. a secondary antibody. In this case specific binding of patient celiac
autoantibodies to the celiac epitope is detected via the replacement of said antibodies by the test
compound and thereby the reduction of the signal measured at the maximum binding.

The invention also relates to kits for performing the diagnostic methods as outlined above.
The kits of the present embodiment can comprise the constituents of the kits as described above with
the difference that instead of or further to a test protein a test compound as defined herein is
comprised in said kit.

In a preferred embodiment of the invention the test compound as defined in the present invention can
be added to known assay kit useful as a Tissue Transglutaminase Antibody Assay useful in the
detection of celiac disease. Thereby, such an assay can be used as a displacement assay, as taught
herein which is useful to exclude false positive findings. The assay shall be performed in the presence
of a test compound of the invention. Either displacement of the test compound by patient
autoantibodies or displacement of patient autoantibodies by the test compound can be monitored
depending on the method of detection. Such assay kits, so called second-generation human tissue
transglutaminase antibody assays are reviewed by van Meensel et al [Clinical Chemistry 50:11 2125-2135 (2004)]. Assay conditions can be optimized starting from the manufacturers’ guidelines, while the procedure basically can be performed as described.

The invention also relates to uses of the test compounds of the subject invention in the diagnosis of a gluten induced autoimmune disease as disclosed herein.

In a preferred embodiment the test compound of the present invention is different from an antibody, antibody fragment or derivative disclosed in any of the following documents: Sblattero et al. Eur J Biochem. 2002 Nov;269(21):5 175-81, Nakachi K et al. J Autoimmun. 2004 Feb;22(1):53-63. Seissler et al. Clin Exp Immunol. 2001 Aug;125(2):216-21], provided that said antibody is capable of binding, preferably specific binding to the main celiac epitope. Alternatively every antibodies, antibody fragments or derivatives disclosed in one or all or any combination of the above publications or the diagnostic use thereof are excluded from the scope of the present invention.

EXAMPLES

EXAMPLE 1

MATERIALS AND METHODS

1.1 Molecular modelling

Residues 1-14, 44-55 and 123-132 were missing in the crystal structure of human TG2 (PDB code: 1KV3) [Liu S et al. Proc Natl Acad Sci U S A. 2002;99(5):2743-7.1 however, the corresponding regions were visible in the TG3 structure (PDB code: IVJJ.) [Ahvazi B et al., EMBO J. 2002;21(9):2055-67., so this was used for modelling the full-length TG2. Homologous model was built by Modeller [Sali A. Blundell TL, J Mol Biol. 1993;234(3):779-815.] using the multiple template option of the program. Graphical analysis was made on Silicon Graphics Fuel workstation using Sybyl program package (Tripos, St. Louis, MO), and a study was made to search for amino acids that are located near enough to each other but belong to different domains. Particularly, the interface of the core domain with the N-terminal (I) domain and the interface of the core domain with the C-terminal (IV) domain were investigated. Preference was given to charged amino acids based on observations with Ca\(^{2+}\)-binding mutants of TG2.

A set of amino acids was suggested by the present Inventors that might take part in the build-up of the celiac epitope, such as:

Arg\(^{19}\), His\(^{22}\), Glu\(^{153}\), Glu\(^{154}\), Arg\(^{156}\), Arg\(^{433}\), Glu\(^{435}\), Met\(^{659}\), Leu\(^{661}\).

It has also been observed that Arg\(^{19}\), Glu\(^{153}\), Met\(^{659}\), are relatively close to each other

Arg\(^{19}\) Glu\(^{153}\) - 12.9 A

Arg\(^{19}\) Met\(^{659}\) - 7.7 A

Glu\(^{153}\) Met\(^{659}\) - 16.8 A
AH these residues are located at the surface of the molecule and were assumed to possibly form a common conformational epitope. Position and effects of changes concerning further amino acids were evaluated as needed by experimental results.

1.2 Generating TG2 mutants

In order to obtain His-tagged human recombinant TG2, DNA construct encoding TG2 gene (pGEX-2T-TG2; Ambrus A et al., 2001) was used as a template in polymerase chain reaction, which was performed with specific primers (according to the Ek/LIC Cloning Kit, Novagen); oligonucleotide primer 1, 5'- gac gac gac aag atg aga att cag ace atg gcc gag gag ctg g - 3', and primer 2, 5'- gag gag aag ccc ggt tga att egg tta gcc ggg gcc aat gat gac - 3'. The His-tagged TG2 was generated by subcloning of PCR-amplified DNA into pET-30 Ek/LIC Vector.

The TG2 mutants were generated according to the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with the His-tagged TG2 construct in pET-30 Ek/LIC Vector (mentioned above) as a template. A pair of oligonucleotide primers containing the desired mutations was designed for the mutants. After the PCR reaction the parental strand was removed by DpnI digestion. Mutations were confirmed by DNA sequencing using the ABI PRISM® 3100-Avant Genetic Analyzer.

Serine was used for replacement instead of the more conventional Ala because all investigated positions were located at the surface of the molecule, and hydrophobic portion introduced by Ala-mutagenesis may cause folding problem.

The primers used for mutagenesis are listed in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Oligonucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FORRl 9ShTG2</td>
<td>5'- get gga gac caa tgg cag cga cca cca cac ggc cg-3'</td>
</tr>
<tr>
<td>REVRI 9ShTG2</td>
<td>5'- cgg ccc tgt ggt tgt cgc tgc cat tgg tct cca gc-3'</td>
</tr>
<tr>
<td>FOREI 53ShTG2</td>
<td>5'- get tgt tac ctg gac teg age gag gag egg cag g-3'</td>
</tr>
<tr>
<td>REVEL 53ShTG2</td>
<td>5'- cct gcc get cct cgc teg aag cca ggt aca acg c-3'</td>
</tr>
<tr>
<td>FORM659ShTG2</td>
<td>5'- cct get gcc get cca cag egg cct cca cca acg gcg gc c-3'</td>
</tr>
<tr>
<td>REVNM659ShTG2</td>
<td>5'- cca get tgg gga gcc cgc tgt gga gcc gca gca gc g-3'</td>
</tr>
<tr>
<td>FORE158LhTG2</td>
<td>5'- gga aga gga ggc gca gca gta tgt cct cac ccc-3'</td>
</tr>
<tr>
<td>REVE158LhTG2</td>
<td>5'- ggg tga gga cat aca acg gcc get cct ccc-3'</td>
</tr>
<tr>
<td>FORE158QhTG2</td>
<td>5'- ggaagagggcgcagcagtgtgctctcc-3'</td>
</tr>
<tr>
<td>REVE158QhTG2</td>
<td>5'- ggtgagctagctctgtgctcgtcctcc-3'</td>
</tr>
<tr>
<td>FORE154KhTG2</td>
<td>5'- cct gga etc gga aaa gga ggc gca gcg gc-3'</td>
</tr>
<tr>
<td>REVE154KhTG2</td>
<td>5'- cct gcc get cct ttg cgg agt cca gc g-3'</td>
</tr>
<tr>
<td>HTGCHZAPDXEEEEXXE2 (Site 4)</td>
<td>5'- ggg tga gga cat act get gcc get get gcg aag tea ggt aca cag c-3'</td>
</tr>
<tr>
<td>HTGCHZAPDXEEEEXXEi (Site 4)</td>
<td>5'- get tgt tac ctg aac teg cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag c-3'</td>
</tr>
</tbody>
</table>
TG2 sequence used was as disclosed in UniProtKB/Swiss-Prot Database, ID: P21980 (TGM2_HUMAN) which is incorporated herein by reference.

Based on said sequence further primers can be prepared by the same method.

1.3 Expression and purification of TG2 proteins

Rosetta 2™ cells (Novagen) were transformed with the expression vectors and grown in LB at 37 °C to an OD$_{600}$ 0.6-0.8. To induce the expression of His-tagged proteins, the cultures were grown for 5h at 20 °C in the presence of 0.3 mM isopropyl β-D-thiogalactoside (IPTG) then the cells were harvested by centrifugation at 4 °C.

Aut purification steps were performed on ice. The cells were resuspended in lysis buffer [50 mM sodium phosphate (pH 7.4), 500 mM NaCl, 5 mM imidazole, 20 mM β-mercaptoethanol, 10% (v/v) glycerol, 1% (v/v) Triton X-100] with 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysis was performed by sonication followed by centrifugation for 35 min at 20000 g. Supernatants were loaded onto a 2 ml Ni Sepharose High Performance column (GE Healthcare Bio-Sciences AB) in the presence of 20 mM imidazole. The column was washed with 80 mL wash buffer [50 mM, sodium phosphate (pH 7.4), 800 mM NaCl, 20 mM imidazole, 20 mM β-mercaptoethanol], then with 40 mL wash2 buffer [50 mM, sodium phosphate (pH 7.4), 500 mM NaCl, 30 mM imidazole, 20 mM β-mercaptoethanol]. The protein was eluted with 12 mL wash2 buffer containing 250 mM imidazole. The eluent was concentrated with Amicon Centricon-YM 50 MW (Millipore) and the buffer was exchanged three times to storing buffer [20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol].

Domain deletion mutants were generated and expressed as described [Korponay-Szabo I et al., J Pediatr Gastroenterol Nutr. 2008;46:253-61].

1.4 Characterization of mutant proteins

A) Western blotting

SDS/PAGE was performed according to standard techniques. TG2 proteins were separated by SDS/PAGE and transferred to Polyvinylidene Fluoride (PVDF) membrane (Millipore). The membrane was blocked with 1% bovine serum albumine (BSA) in 50 mM Tris-buffered saline containing 0.1% (v/v) Tween 20 (TTBS) for 1h at room temperature, followed by incubation with goat polyclonal anti-TG2 antibody (Upstate) diluted 1:20000 in TTBS or with mouse monoclonal anti-TG2 antibody TGI 00 (NeoMarkers) diluted 1:15000 for 1h at room temperature. After extensive washing with TTBS the membrane was incubated with anti-goat antibody or with anti-mouse antibody conjugated with horseradish peroxidase (HRP) (Sigma), 1:30000 in TTBS, for 1h at room temperature. The bands were revealed by Chemiluminescent ECL Detection System (Millipore).

All mutant TG2s could be expressed in bacteria successfully, and there were only slight differences in yield of the protein and in the intensity of the protein bands compared to the wild type (Wt) enzyme (Figure 1A). This phenomenon is probably due to the different properties of the mutants, which could
affect the expression or purification efficiency. The Coomassie brilliant blue-staining of the SDS gel showed purity greater than 80% for all expressed proteins.

B) Transglutaminase activity

Transglutaminase activity was measured with microtiter plate assay based on the incorporation S-(biotinamido) pentylamine into immobilised N,N-dimethylated casein (DMC) [Kiraly R et al, J Autoimmun. 2006;26(4):278-87].

The wells were coated with 4 mg µL N,N-dimethylated casein (Sigma) in 100 mM Tris/HCl pH 8.0 overnight, 4°C. After washing, the wells were blocked with 0.5% milk powder solution for 30 min at room temperature. Then the reaction mixture was added (in a total volume of 200 µL 100 mM Tris/HCl pH 8.0) containing 10 mM dithiothreitol, 1 mM N-(5-aminopentyl)biotinamide (Molecular Probes, Eugene, OR), 5 mM CaCl₂ and 0.5 µg TG2. The reaction was performed at 37°C for 30 min. The plate was washed with 200 mM EDTA pH 8.5 and incubated with 0.42 µg/well streptavidinel-alkaline phosphatase (Sigma). The enzyme reaction was revealed by adding 200 ml of 25mM p-nitrophenyl phosphate (Sigma) and measuring absorbance at 405 nm. Enzyme activity values were obtained from ΔA405/min of colour development between 10 and 30 min. Reaction blanks contained 10 mM EDTA and no added CaCl₂.

Mutant R showed Ca-dependent TGase activity, which was -30% higher than wild type (Wt) transglutaminase (Fig IB). All of the other mutants showed decreased, but measurable TGase activity (Figure IB). Control mutant (D) in which domains I-III-IV were present, but the catalytic core (II) domain was missing, did not show TGase activity.

C) GTPase activity assay

GTPase activity was determined by the charcoal method [Kiraly R et al, J Autoimmun. 2006;26(4):278-87]. The 100 µL reaction mixture contained 2 µg of recombinant wild type or mutant TG2 in 50 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, 9.9 mM GTP and 0.1 mM [g-32P]GTP (3000 Ci:mmol, Institute of Isotopes Ltd., Budapest, Hungary). The reaction was performed at 37°C for 30 min and was stopped with 700 µL of 6% (w/v) activated charcoal in ice-cold 50 mM NaH₂PO₄ pH 7.5. The mixture was centrifuged and released [³²P] P1 was determined by counting of 150 µL samples of the supernatant. Blank was determined without the enzyme.

The specific activity (cleaved pmol GTP/min/mg enzyme) of the Wt enzyme was set to 100%. The GTPase activities of mutant RM, EM and REM were more than two fold higher than the activity of the Wt (Figure IB). The single and the other double mutants showed 24-50%.

D) Fibronectin-binding capacity of the mutants (Fibronectin-TG2 ELISA)

Microtiter plates (ImmuNoPlate Maxisorp, Nunc, Denmark) were coated with 0.3 µg human fibronectin (FBN) (Sigma) diluted in bicarbonate buffer for 1h at room temperature. The plates were washed 3 times with TTBS containing 10 mM EDTA (TTBS+EDTA) and were incubated with 0.8 µg TG2 in TBS containing 5 mM CaCl₂ and 0.1% (v/v) Tween 20 (Ca-TBS-Tween) for 1h at room
temperature. Monoclonal antibody [TG1OO, 1:500 in TTBS+EDTA; (HeoMarkers, Fremont, CA)] was incubated for 1 h at room temperature. Plates were washed and incubated with HRP-conjugated anti-mouse IgG (1:4000, Sigma) for 1 h at room temperature. The colour reaction was developed by adding 100 µL 3,3',5,5'-tetramethylbenzidine substrate (Sigma) and then stopped with 50 µL 1 N H₂SO₄. The absorbance was read at 450 nm. Standard curves were prepared from dilutions of TG1OO monoclonal antibody for each mutant.

The mutants reacted with monoclonal mouse TG1OO antibody in the same extent as Wt (Figure 2.) when bound to FBN. These data support that the mutants possessed proper conformation in order to be used for further measurements with celiac sera.

1.5 Patients

Unless different conditions specifically mentioned serum samples from altogether 216 celiac disease patients aged 0.9-78 years, of them 56 with selective humoral IgA deficiency (total serum IgA <0.05 g/l), were used; all had diagnosis by small bowel biopsy showing at least Marsh grade III villous atrophy. Samples were collected before the treatment and from 22 of them during a follow-up of up to 17 years. 11 subjects initially had preserved small bowel villous architecture but subsequently developed celiac type villous atrophy during prospective follow up (latent cases). Included non-celiac controls had normal small bowel villous architecture.

1.6 Antibodies and single chain variable fragments (ScFvs)

Mouse monoclonal antibody clone Mab885 (Cl. 885A) of Phadia has been deposited according to the Budapest Treaty by Phadia AB (Box 6460 751 37 UPPSALA, Sweden, Visiting address: Rapsgatan 7P 754 50 Uppsala) under Accession number ................., as indicated in the International form at DSMZ - Deutsche Sammlung von Miroorganismen un Zellkulturen GmbH, Inhoffenstr. 7 B, D-38124 Braunschweig, Germany. Fusion partner of Mab885 is SP2/0 (rat) cl. 321B.

Conditions for cultivation of Cl. 885A are as follows:

Culture Medium: F-DMEM + 4 mM L-glutamine + 5% foetal calf serum
Preferred Temperature: 37 °C
Gaseous Phase: 6% CO2
Optimal split ratio: about 1/10.
Long term storage is preferred in F-DMEM + 10% foetal calf serum + 7.5% DMSO, at -150 °C.

1.7 Anti-TG2 ELISA

The ELISA measurements were performed similar to the testing described previously [Sulkanen, S. et al., Gastroenterology 115, 1322-1328 (1998)]. Briefly, microtiter plates (ImmunoPlate Maxisorp, Nunc) were coated with 0.6 µg TG2 in 100 µL of TBS containing 5 mM CaCl₂ (pH 7.4). The plates were washed 3 times with TTBS containing 10 mM EDTA (TTBS+EDTA). AU antibodies were diluted in TTBS+EDTA. Serum samples (diluted 1:200) or monoclonal antibodies (TG1OO, 1:500; NeoMarkers) were incubated for 1 h at room temperature. Plates were washed and incubated either with HRP-conjugated rabbit anti-human IgA or IgG (1:5000; Dako) followed by HRP-conjugated
anti-mouse IgG (1:5000; Sigma) for 1 h at room temperature. The color reaction was developed by adding 100 µL 3,3',5,5'-tetramethylbenzidine substrate (Sigma) and then stopped with 50 µL 1 N H2SO4. The absorbance was read at 450 nm. Standard curves were prepared from dilutions of TGIOO monoclonal antibody for each mutant and binding of other antibodies was calculated by 4-parameter fit if binding to Wt TG2 was 100%. All serum samples were examined in duplicates.

1.8 Competition ELISA assays

Microtiter plates were coated with 0.6 µg or less wild type (Wt) TG2 in 100 µL of TBS containing 5 mM CaC12 (pH 7.4). Wells were incubated with celiac serum (1:800) and increasing amounts of purified total celiac IgG antibodies (dilution 1:200-1:50) or IgA deficient patient serum and binding of IgA and IgG antibodies were detected. In further competition assays celiac serum was added to the plate together with increasing amounts (up to 18 µg/well) of monoclonal mouse antibodies (885, CUB7402, H23) and bound IgA was measured.

1.9 Immunofluorescent studies

Unfixed frozen sections were incubated with anti-human IgA or IgG (DAKO) to detect in vivo-bound immunoglobulins alone or in combination with double labelling for TG2 carried out as described previously [Korponay-Szabo, LR. et al. J. Pediatr. Gastroenterol. Nutr. 31, 520-527 (2000), Korponay-Szabo, LR. et al. Gut 53, 641-648 (2004)]. Detecting competition with TG2-specific MAbs was done by adding MAbs to the tissue for 30 minutes in PBS, removing the incubation solutions and testing them for patient IgA and mouse antibodies. Binding of the MAbs was detected by Alexa-Fluor 594-conjugated or Rhodamine conjugated anti-mouse antibodies.

1.10 HUVEC preparation and cell culture experiments

Human umbilical vein endothelial cells (HUVECs) were prepared from fresh umbilical cords and cultured by standard techniques [Palatka K, et al. World J. Gastroenterol. 12, 1730-1738 (2006)]. For the analysis of antibody effects on cell differentiation, HUVECs were cultured on collagen I for 48 hours [Myrsky, E. et al. Chn. Exp. Immunol. 152, 111-119 (2008)] and length of endothelial tubules was analyzed by Image J software. Ten different pictures were taken from three wells with 50,000 cells/well.

1.11 Statistical analysis

Data from the ELISA measurements were analyzed using GraphPad Prism Software and STATISTICA. For comparison of antibody binding to mutant TG2, data were analyzed using repeated measures ANOVA followed with Dunnett's Multiple post test, one way ANOVA followed by Tukeys post test, or Kruskal-Wallis test followed by Dunn's multiple comparison test as appropriate. A p value < 0.05 was considered significant.

EXAMPLE 2

PRELIMINARY EXPERIMENTS TO LOCALIZE THE CELIAC EPITOPE

Binding of celiac antibodies is related to a calcium binding site of TG2
During examination of Ca\(^{2+}\) binding of human TG2 we identified two negatively charged surface patches on the core domain that are near to each other and could serve as Ca\(^{2+}\) binding sites. Multiple mutations of acidic glutamate and aspartate residues to neutral glutamine and asparagine in Site4 (151DSEEERQE158 → 15 INSQQQRRQQ 158) or Site 5 (434DERED438→434NQRQN438) led to the decrease of the number of bound Ca\(^{2+}\) ions per TG2 molecule from 6 to 3 and also diminished the binding of celiac patient serum samples substantially (Site 4, residual binding compared to wild TG2 11.6±8.5%) or moderately (Site 5, 51.3±16.0%) in enzyme-linked immunoassay (ELISA). These ELISA assays were performed with serum samples from 62 celiac disease patients (age at diagnosis 1-42 years) and 20 disease control subjects (age 1-17 years). The serum samples were collected at the time of initial clinical diagnosis, celiacs having anti-TG2 and endomysial antibodies in serum and having severe villous atrophy by small bowel biopsy, whereas controls having normal small bowel architecture and negative serology results, respectively.

For performing the ELISA, wild-type and mutant TG2 proteins were diluted in Ca-TBS-Tween and then coated to Maxisorp plate (0.6 µg/well) at room temperature for 1 hour. Then the ELISA was conducted as described in example 1, by adding patient antibodies at 1:200 dilutions in assay buffer and using rabbit anti-IgA polyclonal antibodies (DAKO, diluted 1:4000 in assay buffer) to detect the signal. The binding to wild-type TG2 was set to 100%, and binding of patient samples was calculated as proportional to this, using a TG100 monoclonal antibody reference to control that plates have comparable amounts of wild-type and mutant TG2 antigens.

In a liquid phase version of the assay microtiter plates (Maxisorp, Nunc, Denmark) were coated with 0.6 µg wild type (Wt) TG2. Celiac sera were preincubated with different amount of Wt or mutant TG2 in Ca-TBS-Tween for 10 min at room temperature. This mixture was added to the wells and incubated for 1h at room temperature. The coated Wt TG2-bound IgA antibodies were detected similarly as in anti-TG2 ELISA.

In search of the anchor residues that could form a celiac epitope in Site 4, we prepared mutant TG2 molecules bearing D151N, E153Q, E154Q, E155Q, E158Q and E158L mutations separately. From these, mutation of residues 153 or 158 significantly decreased (p<0.0001) the binding of celiac antibodies (Figure 3A) whereas the other changes did not have any effect. As E158 is not surface exposed, these results showed the importance of Glu\(^{153}\), that could form an anchor point for the antibody binding. However, the mutation of Glu to Gln at this position was not sufficient to completely abolish patient antibody binding, or there might be also the possibility, that Glu\(^{153}\) needed cooperativity of other surface parts to form a functional epitope.

'Site-directed mutagenesis was performed also at Site 5. Analyzing surface properties, two candidate amino acids (Arg\(^{333}\) and Glu\(^{335}\)) with charged side-chains in the middle of the putative epitope of Site5 were changed to serine (Mutant 433), because these were the nearest to Site 4. Mutant 433, however, did not show altered binding with celiac disease patient serum samples. In addition, monoclonal mouse anti-TG2 antibody H23 [Korponay-Szabo I et al. J Pediatr Gastroenterol Nutr. 2008;46,253-61] which has its binding epitope at Site 5 did not interfere with celiac disease antibodies in their
binding to wild-type TG2 when co-administered in excess together with celiac disease serum samples in ELISA.

We also prepared a transglutaminase active site mutant (C277S), but this protein bound celiac antibodies similarly well as wild type TG2.

Calcium ions do not form part of the celiac epitope(s)

Site 4 is a Ca\(^{2+}\) binding site and previous clinical laboratory studies indicated that celiac antibodies recognize TG2 preferentially in the presence of Ca\(^{2+}\) [Sulkanen S et al, Gastroenterology 1998;1 15:1322-8]. Since surface amino acids involved in the coordination of Ca\(^{2+}\) at Site 4 or Site 5 were not entirely responsible for celiac antibody binding, we explored whether calcium ions themselves also contribute to the epitope. For this purpose, we utilized TG2 preparations as antigen after exhaustive dialysis in ethylene-diamine-tetraacetate (EDTA) and we used EDTA in all buffer solution in ELISA. This procedure, however, only can remove all bound Ca\(^{2+}\) from the protein when its strong Ca\(^{2+}\) binding site (Site 1, amino acids 229-233, homologous to the strong Ca\(^{2+}\) binding site of TG3) is mutated. This Site 1 mutant where Site 4 and Site 5 aminoacids were intact was a good antigen for celiac antibodies and bound them equally well (>100%) in ELISA both in the presence and absence of Ca\(^{2+}\). This result excludes the structural role of Ca ions in the potential celiac epitope(s) at Site 4 as well as at Site 5.

Existence of epitope anchor points outside the core domain

As Glu\(^{153}\) was near to interfaces between domains, we tried to establish which of the domains other than the core domain might harbour further epitopes or might cooperate with Glu\(^{153}\) in antibody binding. Given that earlier results with truncated TG2 fragments were contradictory in the previous art, we expressed TG2 mutants each lacking one structural domain of TG2 as described [Korponay-Szabo et al, J Pediatr Gastroenterol Nutr. 2008;46:253-61.] When these domain mutant constructs were applied in equimolar concentrations in solid and liquid phase immunoassays, we found that celiac antibody binding proceeded normally even in the complete absence of domain III (aminoacids 472-584). In contrast, lack of either domain I or II resulted in proteins that did not bind celiac antibodies at all (Figure 3B), suggesting that both of these domains have direct or indirect role in antibody binding. Also the loss of domain IV (aminoacids 585-687) affected the binding to some extent (O.D. relative to wild TG2 0.8, pO.0001). However, it was surprisingly also noted, that the construct consisting of domains I-III-IV but not containing domain II did not bind celiac antibodies, even though these domains are known to adopt their folded forms [Hang J et al, J Biol Chem. 2005;280(25):23675-83. ; Pinkas DM et al, PLoS Biol. 2007;5(12):e327.] also independently of the core domain. Thus these results indicated that in the celiac epitope(s) the presence of the core domain anchor residues is needed for efficient binding, and so in their absence the other epitope parts were not able to form functional binding sites.
EXAMPLE 3

EFFECTS OF MUTATIONS IN THE PUTATIVE CELIAC EPITOPE AND RELATED SURFACE AREAS

Based on the preliminary results and computational analysis, within a larger set of amino acids 6 amino acids (R19, D151, E153, E154, E155, M659) were identified that are on or adjacent to the surface of TG2 related to Site 4 and close enough to each other to potentially form composite epitopes. These residues were changed one by one (single mutants) or in combination (double and triple mutants) by site-directed mutagenesis to serine or in case of acidic residues to their neutral homologues.

We prepared the following point mutant TG2 molecules: E153S (E), R19S (R), M659S (M) or the respective mutations in combination (D151N/E154Q/E155Q, RE, EM, RM, REM, E154K/R19S/M659S [RKM]), and tested the proteins with a large set of consecutive patient serum samples (n=76). Relative amounts of bound antibodies were calculated by comparison to a calibrator curve constructed from the concentration dependent binding of mouse monoclonal anti-TG2 antibody TG1 00, which has a linear epitope at amino acids 447-538.

Each single mutation resulted in significant decrease in celiac antibody binding (26.5%, 28.8% and 39.1% remaining binding for R, E and M, respectively) and double and triple mutations caused proportionally changes. The D151N/E154Q/E155Q mutant also bound significantly less antibodies than its single point mutants (Figure 3A), but did not lose completely its binding ability. The RM mutant still retained 35.8% binding capacity, EM had 18.5% and REM and, surprisingly, RE were the lowest, giving 13.4% and 6.6% binding, respectively. The same binding pattern was seen both for consecutively diagnosed childhood and adult celiac patients (Figure 4), which results together pointed out the importance of the first alpha helix of the core domain in antibody binding.

Despite of the decreased binding of celiac antibodies, these mutant TG2 proteins bound normally a large set of mouse monoclonal anti-TG2 antibodies [Korponay-Szabo I et al, J Pediatr Gastroenterol Nutr. 2008;46:253-61]. showed functionality in fibronectin binding, and in either TG2-ase or GTPase assays, as shown in Example 1. Further, CD spectra analysis of selected mutants (R, S4, E153 and E158 mutants) showed that the proteins had a folded structure without significant increase (<5%) in the proportion of unordered segments.

Direct binding of celiac antibodies to the surface around Glu153 was further corroborated by modifying another core domain anchor point of the putative composite epitope. As shown above, changing Glu154 to neutral Gln (E154Q) was not sufficient to alter celiac antibody binding, but change in TG2 (E154K) in combination with the R and M mutations (RKM) resulted in similarly dramatic reduction of celiac antibody binding (remaining binding 22.0%) as the REM mutation (Figure 5).

This result is also supported by the fact that Factor XIII, one other member of transglutaminase family to which celiac antibodies do not bind [Sjöber et al, Autoimmunity 2002;35:357-64.] contains a positively charged lysine at the corresponding position within an otherwise very homologous surface.
In contrast, library search of TG2 sequences from different species and human TGs showed that all animal TG2 proteins that are known to serve as highly sensitive antigens for the detection celiac disease antibodies in clinical laboratories by ELISA (guinea pig) or traditional immunofluorescent assays with tissue sections (monkey, rat, rabbit, mouse) do contain homologous negatively charged surfaces and anchor points at the corresponding positions (Table 2). A similar surface also can be found in human TG6.

To delineate the surface area that could contribute to the putative celiac epitope, we used the data obtained with Mutant 433 (Figure 4), which shows that the putative celiac epitope does not extend to these residues in direction of Site 5.

Table 2. Comparison of amino acids contributing to the celiac epitope in TG2 proteins from different species and other members of the transglutaminase superfamily

<table>
<thead>
<tr>
<th></th>
<th>Anti-</th>
<th>19</th>
<th>151</th>
<th>152</th>
<th>153</th>
<th>154</th>
<th>155</th>
<th>156</th>
<th>157</th>
<th>158</th>
<th>431</th>
<th>433</th>
<th>435</th>
<th>659</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG2_Human</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG2_Monkey</td>
<td>High*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG2_Guinea pig</td>
<td>High*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG2_Rat</td>
<td>High*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG2_Mouse</td>
<td>High*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG6_Human</td>
<td>Medium</td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>TG3_Human</td>
<td>Low*</td>
<td>Q</td>
<td>G</td>
<td>N</td>
<td>H</td>
<td>A</td>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td>S</td>
<td>A</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>FXIII_Human</td>
<td>Low*</td>
<td>V</td>
<td>N</td>
<td>K</td>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G</td>
<td>G</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>EPB_Human</td>
<td>Low*</td>
<td>E</td>
<td>K</td>
<td>N</td>
<td>A</td>
<td>Q</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td>S</td>
<td>R</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>TG1_Human</td>
<td>Low*</td>
<td>H</td>
<td>W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S</td>
<td>M</td>
<td>P</td>
<td></td>
</tr>
</tbody>
</table>

b) [Dieterich et al, Nat Med. 1997;3:797-801.]
c) [Korponay-Szabo et al, Gut. 2003;52:199-204.]
d) [Hadjivassiliou et al, Ann Neurol. 2008;64:332-43.]
g) Erythrocyte Band 4.2 protein, unpublished own results
Two anchor points sufficiently determine the celiac epitope.

To establish the relative importance of Arg\textsuperscript{19} and Met\textsuperscript{659} in celiac antibody binding, further studies were conducted with fibronectin-bound TG2 that mimics accessibility of epitopes in the extracellular matrix where antibodies primarily meet the autoantigen in patients. For this, 0.8 μg TG2/well was coated to fibronectin as described in Example 1, and bound IgA class celiac antibodies were detected by anti-IgA secondary rabbit antibodies (DAKO) diluted 1:4000 in assay buffer. Under these conditions, the mutations of Arg\textsuperscript{19} and Glu\textsuperscript{153} had similar effects as when tested with TG2 directly bound to the ELISA plate. Surprisingly, the mutation of Met\textsuperscript{659} alone, however, did not alter celiac antibody binding in either children or adults (Figure 6), nor had additive effect if used combined with the other two mutations. Thus these results show that of Glu\textsuperscript{153} and Arg\textsuperscript{19} together are enough for antibody binding, and thus celiac antibodies may bind to TG2 also in its catalytically active form when TG2 adopts an open-extended conformation [Pinkas DM et al, PLoS Biol. 2007;5(12):e327.] where domain IV with Met\textsuperscript{659} swings out. This is even more surprising as Arg\textsuperscript{19} is significantly closer in the closed conformation to Met\textsuperscript{659} (7.7 A) than to Glu\textsuperscript{153} (12.9 A). On the other hand, measurements on the position of Arg\textsuperscript{19} and Glu\textsuperscript{153} in the open form crystal structure (2Q3Z) did not show difference compared to the closed conformation (1KV3, 2.8-A ) (Figure 7).

In liquid phase assay antibodies showed the same pattern as in solid phase ELISA supporting the finding that Glu\textsuperscript{153} and Arg\textsuperscript{19} has major role in the antibody-binding.

**EXAMPLE 4**

**OTHER MUTATIONS INDIRECTLY AFFECTING THE CELIAC EPITOPE.**

As shown in Figure 3A, a single point mutation on the core domain (E158Q or E158L) also severely reduced the binding of celiac autoantibodies to TG2 in ELISA. As this amino acid is not surface exposed, we conducted further in silico analysis and molecular modelling to explore the effect of this mutation. Glu\textsuperscript{158} is located at the base of the first alpha helix of the core domain, and loss of the negative charge on its side-chain results in the disruption of hydrogen bonds and confers instability to the helix. This process may affect the relative position of the surface residues 153, 154 and 155 to each other or to the Arg\textsuperscript{19} on the N-terminal domain.

Evaluating also the position of the amino acids of Site 5, it was concluded that also these changes may affect the helix of Site 4 in indirect ways, but to a lesser degree.

Further, we evaluated whether changes other than the mutation of Arg\textsuperscript{19} in the N-terminal domain first alpha helix would have an effect on celiac antibody binding. We thus prepared mutant TG2 where the N-terminal first 14 amino acids of full length TG2 were deleted, that was supposed to affect also the said helix. Celiac antibodies showed a severely reduced binding to this mutant (8.4% residual binding compared to wild type TG2.

It is true for all these mutated amino acids that they do not form part of the molecular surface of the celiac epitope, however, a mutation indirectly effects said epitope.
EXAMPLE 5
DISEASE SPECIFICITY AND CLINICAL RELEVANCE OF THE COMPOSITE EPITOPE

Disease specificity

We evaluated whether the identified composite epitope is characteristic only for celiac autoimmunity. Therefore we compared the binding pattern of 11 celiac disease samples which were positive for both endomysial and anti-TG2 antibodies with that of serum samples from 11 patients with anti-TG2 antibodies in serum due to other autoimmune diseases (SLE, Sjögren's syndrome, rheumatoid arthritis). The binding pattern of the non-celiac group was clearly different and unrelated to the celiac epitope (Figure 8), and these subjects were also negative for antibodies against endomysium and deamidated gliadins. The autoimmune patients did not have malabsorption or other intestinal symptoms, and when a small bowel histology was performed (in 1 case), the small bowel was normal. Antibodies of different celiac patients recognize parts of the same epitope. Since natural patient antibodies are polyclonal and may contain a mixture of antibodies with different epitope specificity, we next investigated whether the newly identified conformational epitope is determining antibody response in all celiac cases. Therefore we conducted competition studies with serum samples and purified IgG and IgA patient immunoglobulins. Serum samples from all investigated 56 IgA deficient celiac patients (aged 0.9-78 years) with IgG or IgM class anti-TG2 antibodies were able to displace the same IgA anti-TG2 celiac antibody from the same IgA competent celiac patient (Figure 9) while sera of 23 non-celiac IgA deficient control subjects without anti-TG2 antibodies were ineffective. The displacement effect was proportional to the concentration of IgG and IgM class anti-TG2 antibodies in the sera and was thus suitable to measure unknown non-IgA celiac antibodies in the clinical laboratory. Although all celiac patient serum samples displayed a severely reduced reaction with REM, RKM triple and RE double mutants, their reaction showed some variability with the R point mutant where only the N-terminal anchor point (Arg^{39}) had been changed as shown in Figure 4. However, when we performed the competition studies with purified IgG antibodies from a celiac patient whose antibodies did react with the R, we got the same competition effect for the same IgA celiac antibody as when we used other IgG celiac antibody that did not react with R (Figure 9). Further, when we compared the antibody binding results to the Site 4 and Site 5 Ca^{2+}-binding mutants obtained with the originally investigated 62 celiac serum samples, the binding to Site 5 correlated with the logarithmic values of the binding to Site 4 for the individual samples (Figure 10). This result indicates that the binding was primarily determined by the Site 4 helix but also changes at the nearby Site 5, that is at the margin of the Site 4 surface area and can influence the position of the helix of Site 4, had proportional effect pointing to the existence of a single main binding site for all patients. This series contained also adults, for whom similar results were observed.
Stability and evolution of the epitope binding pattern during disease development

Celiac disease is a strictly gluten-dependent clinical entity and autoantibody production to TG2 is dependent on gluten ingestion, disappears and reappears with gluten elimination and reintroduction. Therefore, we investigated the epitope specificity of circulating TG2 autoantibodies of individual patients from the initially obtained and subsequent serum samples. In a series of 7 patients, in whom antibodies disappeared on diet, but gluten was reintroduced at a later time (after 1-4 years) for diagnostic gluten challenge, as used to be clinical practice in earlier times, the patients responded with antibodies of the same epitope specificity on gluten challenge. Similarly, in cases without diet compliance and constantly positive for TG2 antibodies in serum over a long time from childhood into adulthood, stability of the epitope binding pattern was observed for 3.5-14 years (Figure 11).

Similar epitope specificity of circulating antibodies and of those in vivo-bound to patient tissues

Next we evaluated whether antibodies to the composite epitope are also relevant for the clinical disease manifestations. There are several clinical observations that high avidity antibodies might be trapped in the tissues and do not circulate, explaining seronegative celiac cases in the minority of patients [Salmi TT et al, Gut. 2006;55(12):1746-53]. The celiac antibodies deposited in tissues were present all diseased organs organs [Korponay-Szabo IR et al, Gut. 2004;53(5):641-8] and were shown to be functional as they bound externally added recombinant TG2 [Salmi TT et al, Gut. 2006;55(12):1746-53], thus they might be more important for disease pathogenesis that blood antibodies. Therefore, we aimed to test whether epitope specificity of tissue-bound TG2 antibodies is similar to blood antibodies. We eluted patient IgA antibodies deposited in patient tissues from tissue sections and tested them with the panel of relevant mutant TG2 proteins after purification. In order to obtain only the functional patient antibodies that bound to the TG2 antigen and to avoid contamination of the IgA fraction by nonspecific IgA contained within plasma cells or epithelial cells that does not necessarily take part in the disease process, we chose not to use gut biopsy samples but an extraintestinal organ without local production of IgA. Such a patient tissue was easily available in sufficient amounts for biochemical studies without performing invasive procedures when we tested placenta samples from two celiac mothers who gave birth while having serologically active disease. In fact, we observed that placenta in celiac disease contains high amounts of TG2-bound maternal antibodies both in the decidual parts and on the surface of the chorionic villous structures (Figure 12). This IgA was eluted from the placenta tissues with chloroacetic acid as previously described [Korponay-Szabo I et al, Gut. 2004;53(5):64 1-8.9], and showed identical epitope targeting pattern to the typical pattern observed with celiac serum samples.

Celiac antibodies targeted against the identified composite epitope cause disease in passive transfer

To assess whether the antibodies targeting the celiac epitope we identified are involved in disease phenomena, we sought a disease model where humoral and cellular components of the immune reaction could separately be investigated. Unfortunately, celiac disease is a strictly human disorder and there aren't relevant animal models where TG2-specific antibodies would be operative. Thus we explored whether natural transfer of maternal antibodies occurs in human newborns and whether this
process is accompanied by pathological features. These offsprings are often underweight and have more often obstetrical complications than children of normal mothers [Hadziselimovic F, et al. Fetal Pediatr Pathol. 2007;26:125-34]. Therefore, we investigated placenta, umbilical cord and serum specimens at 8 deliveries where the mother had celiac disease and human umbilical cord endothelial cells (HUVEC) were prepared. Three of these mothers had active disease with circulating TG2 antibodies with the typical specificity as assessed by ELISA with their serum samples. One of these mothers was IgA deficient with high serum levels of IgG class antibodies. IgG class anti-TG2 antibodies appeared in all three infants' serum. These antibodies had similar epitope specificity as the IgG antibodies of their mothers (Figure 12). The IgG antibodies were detected also in the umbilical cord tissue of the newborn from the IgA deficient mother, and this baby was underweight and had liver damage for which no other clinical cause was found and which resolved in parallel to the recession of serum antibody levels in blood. Jejunal biopsy was not possible in this infant by ethical reasons. HUVEC cells prepared from another baby exposed to maternal antibodies before birth displayed reduced cell survival, abnormalities in their shape and spreading on surfaces compared to cells from babies with antibody-negative celiac mothers.

EXAMPLE 6

DESIGN OF DIAGNOSTIC TRANSGLUTAMINASE MUTANTS OTHER THAN TG2

Using molecular modelling, we evaluated the structure of transglutaminase family member proteins and their surfaces corresponding to the composite celiac epitope identified in TG2. As shown in Table 2, the first N-terminal helix of the core domain is similar also in Factor XIII and TG3, which, however differ in some of the anchor points of the celiac epitope and typically do not cross-react with the classic celiac autoantibodies. Given the similarities in the the overall structure framework of these molecules, we expect that changing the amino acids corresponding to the main anchors points of the celiac epitope will enhance their binding properties. Based on results from molecular modelling and our experimental data with the E154K mutation in TG2, change of XIII factor Lys^{199} to Glu is predicted to increase celiac antigenicity of Factor XIII (Figure 5). This engineered TG antigen could be used as reference protein together with original Factor XIII as test protein for measuring patient samples in a celiac epitope-specific way in ELISA or other immunoassay. In this embodiment, signal with the engineered protein but absence of the signal with original Factor XIII would indicate a celiac-type antibody binding. Similarly, change of Gly^{146} and/or His^{148} in TG3 or corresponding amino acids in other human or eukaryote TG family members could have a similar effect.
EXAMPLE 7

DEVELOPMENT OF DIAGNOSTIC KITS

Diagnostic kit based on mutant TG2

A diagnostic kit can be developed based on the specific celiac antibody binding site of TG2 and the assay conditions described in examples 1-3. This kit is based on ELISA method, other immunoassays, or label-free binding assays. In ELISA setting, wild type TG2 and one or more mutant TG2s with an altered celiac epitope are bound to the surface of the plate and the binding pattern of the investigated sample would distinguish between false-positive and "real" celiac sera. The TG2 protein with the intact celiac epitope will be the reference and the decrease of antibody binding with celiac epitope-specific mutants indicates the celiac-type binding pattern. If a binding is detected to the reference protein but without at least a 30% change in the binding of the test protein, the result is interpreted as non-celiac type TG2 antibody result and no further clinical investigations are performed. In this way, unnecessary invasive investigations, like upper endoscopy and small intestinal biopsy can be avoided. If the results with both the reference and the test protein come out as lower than a predetermined cut-off level, the sample does not contain TG2 antibodies and thus invasive investigations can be avoided as well. A suitable cut-off for declaring antibodies as reactive with the reference TG2 protein are established by a receiver operating characteristic curve (ROC) performed with known celiac and non-celiac samples. A cut-off of 5 % of the binding obtained with a sample from patient with untreated celiac disease is suggested based on results in example 5.

In addition to full-length wild type TG2, our following recombinant proteins showed a binding similar to wild-type TG2 and are suitable to be used as reference proteins in the above assay: D151N, E154Q, E155Q, C277S, Mutant 433, the domain mutants B (domains I-II-IV) and A (domains I-II-III) and the Sitel Ca^{2+}-binding mutant with or without Ca^{2+} ions. The latter is useful in assay settings were addition of calcium would be undesirable. Further, a reference protein could be an engineered protein with a built-up celiac epitope on a TG family framework that is non-antigenic per se for celiac antibodies. For a test protein, mutants R, E, E158Q, E158L, but more preferably combined mutants RE, REM, RKM, Site 4 mutant, or the homologous TG protein without a celiac binding epitope representing the counterpart of the engineered protein are suggested.

Figure 13 shows an example for label-free measurement of celiac antibody binding with Biacore method, where both binding and dissociation is altered of celiac antibodies to Site 4 mutant compared to wild TG2.

Diagnostic kit based on antibody displacement

It was found that natural IgA and IgG antibodies purified from sera of celiac patients and recognizing R19S differently also competed with each other but not with non-celiac human IgG (Fig. 9 B1 to B3). Based on this finding we developed a diagnostic ELISA with 98% sensitivity and 96% specificity for measuring IgG class celiac antibodies in subjects with selective humoral IgA deficiency by their
concentration-dependent displacement effect (r=0.88) on a known celiac IgA tracer antibody (Fig.9 A1 to A2).
In this exemplary assay we measured serum IgG class anti-TG2 antibodies in IaA deficient patients by displacement of an IgA celiac patient antibody. Serum samples from 56 untreated celiac disease patients aged 0.9-73 years (median 10.5) with selective IgA deficiency (total serum IgA <0.05 g/l), from 23 non-celiac IgA deficient and 22 normal serum IgA controls with normal small bowel architecture (age of the controls 1-18 years, median 5.5) were measured in ELISA with wild-type TG2. While the majority of the patients had IgG class patient antibodies, three of them also had IgM class anti-TG2. Investigated serum samples were diluted 1:100 in assay buffer containing a celiac IgA tracer antibody diluted 1:500 and the binding of the IgA antibody was measured by anti-IgA peroxidase-conjugated secondary antibodies. The optical density values (OD) obtained with blank were defined as 100% inhibition and the signal with the IgA tracer antibody without added IgA deficient samples was defined as 0% inhibition. The inhibition obtained with the investigated samples was calculated as 100-(ODtracer-ODsample / ODtracer). Intra assay variability was 5.9% and inter assay variability was 9.2%. The optimal cut-off as a predetermined threshold calculated from receiver operated characteristics curves was 11% inhibition where the displacement assay had 98.2% sensitivity (95% confidence interval: 94.5-100) and 95.6% specificity (95% confidence interval: 89.9-100) for the diagnosis of celiac disease in IgA deficient persons. (Fig 9.A1) Correlation of the displacement effect with the serum concentration of IgG anti-TG2 antibodies measured by direct recognition of human IgG by monoclonal mouse anti-human IgG (Phadia) and anti-mouse secondary HRP-conjugated antibodies; r=0.88. (Fig 9.A2) In the present experimental setting the most preferred displacement ratio was observed at IgG dilution 1:100 and IgA dilution 1:500.

EXAMPLE 8

PREDICTION OF FORTHCOMING CLINICAL DISEASE BY THE EPITOPE SPECIFICITY OF PATIENT ANTIBODIES
In order to see whether the reaction to the identified composite celiac epitope is already seen at the early preclinical stage of the disease and has also predictive value for diagnosis of celiac disease n later life, we tested 11 serum samples from patients positive for endomysial and TG2 antibodies in whom at the time of the serum collection signs of intestinal disease were not present (normal villous architecture) and thus the diagnosis of celiac disease could not immediately be made. Six of these subjects were symptom-free family members of known celiac disease patients and were picked by family screening. During a follow-up of 0.8-3 years, seven of these subjects developed severe villous atrophy in the small bowel with local antibody deposition and further two had antibody deposition with other minor lesions. The serum samples collected at the latent stage of the disease showed in all 11 cases similar epitope recognition pattern as the serum samples of celiac patients with overt clinical disease (Figure 14), and the difference between the reaction to the wild type TG2 and mutants RE and REM was even bigger.
Further cases giving diagnostic challenge are subjects in whom TG2 antibodies do not circulate. In such cases, the antibodies may be trapped within the tissues, and be available for diagnosis in the form of eluates. As these cases often present with extraintestinal symptoms that can be induced also by other diseases, epitope specificity will help to establish whether the observed pathology is gluten-related or not.

EXAMPLE 9
PREPARING DIAGNOSTIC MONOCLONAL ANTIBODIES BY THE PHAGE DISPLAY METHOD

A further tool to produce monoclonal antibodies that target the celiac epitope is creating a library from the variable regions of human antibodies by phage display technique. During this method, single chain variable fragments (scFv) are cloned from human samples, produced and selected for a given antigen [Marzari, R. et al. J. Immunol. 166, 4170-4176 (2001)]. There is a chance to find a TG2 specific antibody having the same binding site than the celiac antibodies from symptomatic patients.

The first step in the process of total antibody library preparation is total RNA isolation by Trizol from intestinal lymphocytes. Next, first-strand cDNA is synthesized using the isolated RNA as template and the variable region of H, K and \( \lambda \) chains of IgA antibodies are amplified with specific primers by PCR technique. After the addition of a linker region, the amplified variable regions of heavy (VH+linker) and light chains (Vk+linker and \( \lambda + \)linker) are assembled by another PCR. For the library production the assembled VH-VK and VH-\( \lambda \) are digested with restriction enzymes and ligated to the pDAN5 vector digested with the same enzymes. After this step the vector contains the assembled scFv as an insert and it can be transformed to DH5\( \Delta F^\prime \) electrocompetent E. coli cells by electroporation. The bacteria which contain the antibody library are grown overnight on selective media and next day, the library is harvested and stored at -80 C. The library should contain ~10^7 clones to maintain variability comparable with variability of antibodies in the human body.

Selection of the library for a given antigen is performed by phage display technique. For this, the bacteria containing the library are grown in selective medium and infected with helper phage. The helper phage provides enzymes and other proteins for the assembly of new, recombinant phages, which contain the scFv fused to its coating protein p3. After the production, the recombinant phages reacting with TG2 are selected with TG2 protein antigen coated immunotubes that have a Maxisorp (DAKO) surface. This antigen contains an intact celiac epitope. The uncoated surface is blocked with buffer containing bovine serum albumin, and the phages are added to the immunotube with the same blocking solution. Phages, which contain TG2-specific scFv on their surface, will bind to the coated antigen. These phages are then eluted from the immunotube with DH5\( \Delta F^\prime \) bacteria, grown overnight in plated bacteria, and harvested the next day as the 1st output of selection. Second and more selection rounds are performed (growing bacteria from the previous output and with the same steps as in the first round) to enhance specificity of the clones, using TG2 proteins - preferably our E or RE mutants - were the celiac epitope is altered, and then growing only the bacteria which contain inserts specific
for the intact TG2 but not for the TG2 protein with the altered celiac epitope. In this way, clones containing antibodies targeted against the desired TG2 epitope are specifically selected and grown further on selective media, while clones reacting to other epitopes of TG2 are discarded. The epitope specificity of the clones is verified by ELISA using the produced antibodies, characterized by fingerprinting PCR and digestion with restriction enzymes. ELISA plates are covered with full length human recombinant TG2 and mutant TG2 proteins and then incubated with the phages. The binding is detected with anti-phage M13 antibody conjugated with peroxidase (GE Healthcare), revealed with tetramethylbenzidine and the reaction is stopped with sulphuric acid. The absorbance values are read at OD450 by spectrophotometer.

**EXAMPLE 10**

**COMPETITION ELISA FOR EVALUATING DIAGNOSTIC PATIENT SAMPLES FOR EPITOPE SPECIFICITY**

Antibodies against TG2 in patient samples may cause diagnostic difficulties because either the detection of their antibody class might be difficult (like IgM) or their epitope specificity is uncertain. To obtain a disease-specific antibody result, we designed two kinds of competition ELISA where the binding of patient antibodies to a TG2 antigen with intact celiac epitope was evaluated in the presence and absence of a test antibody interfering with the patient antibody-celiac antigen binding at the level of the epitope. For the ELISA application, the isotype of the test antibody was different than the antibody to be measured.

a) In the first type of ELISA, the monoclonal antibody 885 having a partially overlapping epitope as patient antibodies was used as the competitor test antibody in excess and the remaining binding of the celiac IgA patient antibodies to wild-type TG2 was measured as described in Example 9. Before adding 885 plus patient antibodies together, an optimising experiment was performed. The amount of TG2 coated on the plate was minimized to still obtain with the given dilution of the patient antibody a signal around O.D. 0.8-1.0 to which the decrease could be compared. We showed that under these conditions the displacement effect was dose dependently 90.6%, 85%, 64% with decreasing amounts of the 885 and in relation to the same patient sample, thus in the presence of 885 only 9.3%, 15% and 36% of patient antibodies bound. This ELISA in one embodiment may also show that if the patient antibody is mostly displaced by 885 or by a similar test antibody, the epitope specificity of the investigated patient antibody is such that it targets the main composite celiac epitope we identified.

b) If the ELISA is performed in the way that the amount of the test antibody is kept constant and the bound amounts of the test antibody are measured by the HRP-conjugate, a decrease in the signal proves the presence of competing TG2-specific antibodies in the sample having the celiac-type epitope specificity. Thereby e.g. unknown patient antibodies with IgG, IgM or IgA framework can be measured at the same time simultaneously by using the 885 test antibody in high dilutions, whereas patients' antibodies are in excess. Using appropriately diluted 885 antibodies, we could achieve that in immunofluorescent test with normal tissue samples patient IgA bound to the TG2 antigen in the
endomysium while signal for the binding of Mab 885 has decreased (Figure 16). This type of ELISA could also be performed using a known IgA class celiac patient antibody and measuring IgG class celiac-specific antibodies of IgA deficient patients as described in Example 5. However, competition between two IgA class celiac antibodies also could be shown if the test antibody is labelled (e.g. biotinylated) and the label is specifically recognized.
CLAIMS

1. A method for diagnosis of a gluten-induced autoimmune disease in a subject comprising the steps of
   i) providing a biological sample taken from a subject, said sample containing autoantibodies of
   said subject, and optionally isolating autoantibodies from said sample,
   ii) contacting the autoantibodies of said sample with
      - a reference protein belonging to the transglutaminase family and having an intact main
        celiac epitope,
      - at least one test protein belonging to the transglutaminase family, in which the side
        chain and/or spatial position of at least one surface amino acid residue contributing to the
        main celiac epitope is altered as compared to the reference protein in which the main
        celiac epitope is intact,
   wherein the said at least one surface amino acid residue of the main celiac epitope
   comprises or is selected from
   - a surface amino acid residue of the first alpha helix of the core domain,
   - preferably the first amino acid residue of said alpha helix, and/or
   - a surface amino acid residue of the first alpha helix of the beta-sandwich domain
   and a surface amino acid of the conserved HisHisThr motif of the beta sandwich
   domain, preferably the sixth amino acid residue of said helix and/or the first
   amino acid of said HisHisThr motif,
   and wherein the core domain has a folded three dimensional structure,
   iii) assessing a binding property of the autoantibodies to the reference protein and to the at least one
   test protein,
   wherein if the binding of autoantibodies to the test protein is impaired as compared to the reference
   protein, this fact is considered as indicative of a gluten-induced autoimmune disease in said subject.

2. The diagnostic method for the diagnosis of a gluten-induced autoimmune disease in a subject
   according to claim 1, wherein in step iii) the binding is assessed by measuring binding level of the
   autoantibodies to the test protein and to the reference protein,
   wherein if the mean binding level of the autoantibodies to the reference protein exceeds a
   predetermined threshold value and if the mean binding level of the autoantibodies to the test protein is
   significantly lower, preferably reduced by at least 30% as compared to the reference protein, this fact
   is considered as indicative of a gluten-induced autoimmune disease in said subject.

3. The diagnostic method according to any of claims 1 or 2, wherein
the first amino acid residue of said first alpha helix of the core domain is an amino acid residue corresponding to or equivalent to Glu53 numbered according to the amino acid numbering of the full length human TG2 based on amino acid sequence alignment, and/or

the sixth amino acid residue of said first alpha helix of the beta-sandwich domain is an amino acid residue corresponding to or equivalent to Arg19 numbered according to the amino acid numbering of the full length human TG2 based on amino acid sequence alignment, and/or

the first amino acid residue of the HisHisThr motif of said first alpha helix of the beta-sandwich domain is an amino acid residue corresponding to or equivalent to His22 numbered according to the amino acid numbering of the full length human TG2 based on amino acid sequence alignment,

wherein preferably

a) the test protein is a mutant transglutaminase (TG), preferably a mutant TG2, TG3 or TG6, and

b) the reference protein is a wild type TG, preferably a wild type TG2, TG3 or TG6.

4. The diagnostic method according to any of claims 1 to 3 wherein in said test protein belonging to the transglutaminase family the side chain or spatial position of at least one further amino acid is altered as compared to the reference protein in which the main celiac epitope is intact,

wherein preferably said further amino acid is selected from the group of amino acid residues corresponding to or equivalent to Arg151, Glu153, Glu154, Arg156, Arg19, His22, Val431, Arg433, Glu435, Met659, Leu661 numbered according to the amino acid numbering of the full length human TG2 based on multiple sequence alignment.

5. A use of a test protein as defined in any of claims 1 to 4 in a method for diagnosis of celiac disease, said test protein belonging to the transglutaminase family, in which the side chain and/or spatial position of at least one surface amino acid residue contributing to the main celiac epitope is altered as compared to the reference protein in which the main celiac epitope is intact,

wherein the said at least one surface amino acid residue of the main celiac epitope comprises or is selected from

a surface amino acid residue of the first alpha helix of the core domain, and/or

a surface amino acid residue of the first alpha helix of the beta-sandwich domain and a surface amino acid residue of the conserved HisHisThr motif of the beta sandwich domain,

and wherein the core domain has a folded three dimensional structure.

6. A diagnostic method for the diagnosis of a gluten-induced autoimmune disease in a subject comprising the steps of
i) providing a biological sample taken from a subject, said sample containing autoantibodies of said subject and optionally isolating autoantibodies from said sample,

ii) contacting autoantibodies of said sample with a protein belonging to the transglutaminase family, said protein having an intact main celiac epitope and

iii) assessing the level of binding of the autoantibodies to the TG family protein both in the absence of and in the presence of a test compound, said test compound known to be capable of binding to the main celiac epitope, at least partially formed or contributed by

one or more surface amino acid residue(s) of the first alpha helix of the core domain and/or

one or more surface amino acid residue(s) of the first alpha helix of the beta-sandwich domain

and the conserved HisHisThr motif of the beta sandwich domain;

wherein if the mean binding level of the autoantibodies in the absence of the test compound exceeds a predetermined threshold value and if the mean binding level of the autoantibodies to the reference protein in the presence of the test compound is significantly lower, preferably reduced by at least 50% as compared to the binding level of the autoantibodies in the absence of said test antibody, this fact is considered as indicative of a gluten-induced autoimmune disease in said subject.

7. A diagnostic method for the diagnosis of a gluten-induced autoimmune disease in a subject comprising the steps of

i) providing a biological sample taken from a subject,

ii) contacting a test compound with a protein belonging to the transglutaminase family, said protein having an intact main celiac epitope and said test compound known to be capable of binding to the main celiac epitope at least partially formed or contributed by

one or more surface amino acid residue(s) of the first alpha helix of the core domain and/or

one or more surface amino acid residue(s) of the first alpha helix of the beta-sandwich domain

and of the conserved HisHisThr motif of the beta sandwich domain;

iii) assessing the level of binding of the test compound to the TG family protein both in the absence of and in the presence of said biological sample,

wherein if the mean binding level of the test compound to the reference protein in the presence of the sample is lower than the binding level in the absence of the sample, this fact is indicative of the presence of autoantibodies capable of binding to the main celiac epitope and of a gluten-induced autoimmune disease in said subject.

8. Use of a test compound known to be capable of binding to the main celiac epitope at least partially formed or contributed by

one or more surface amino acid residue(s) of the first alpha helix of the core domain and/or
one or more surface amino acid residue(s) of the first alpha helix of the beta-sandwich domain and the conserved HisHisThr motif of the beta sandwich domain; in a diagnostic method for the diagnosis of a gluten-induced autoimmune disease in a subject.

9. The diagnostic method of claim 6 or 7 or the use of claim 8 wherein the test compound is a test antibody or antibody fragment, variant or analogue known to be capable of binding to the main celiac epitope of a protein belonging to the transglutaminase family, said protein being an autoantigen in celiac disease, wherein preferably said compound being Mab885 or a fragment or derivative thereof capable of binding to the same epitope region.

10. A diagnostic kit for the diagnosis of a gluten-induced autoimmune disease in a subject comprising

a) a test protein belonging to the transglutaminase family as defined in any of claims 1 to 4, in which the side chain and/or spatial position of at least one surface amino acid residue contributing to the main celiac epitope is altered as compared to the reference protein in which the main celiac epitope is intact,

wherein the said at least one surface amino acid residue of the main celiac epitope comprises or is selected from

- a surface amino acid residue of the first alpha helix of the core domain,
- a surface amino acid residue of the first alpha helix of the beta-sandwich domain
- a surface amino acid residue of the conserved HisHisThr motif of the beta sandwich domain,

and wherein the core domain has a folded three dimensional structure, and

b) a reference protein belonging to the transglutaminase family, said protein having an intact main celiac epitope, and/or a medium carrying instructions for providing and using a reference protein belonging to the transglutaminase family, said protein having an intact main celiac epitope and optionally means for taking or processing a biological sample from a subject, said sample containing autoantibodies of said subject and/or

means for isolating autoantibodies from said sample, and/or

means for assessing the level of binding and/or kinetics of the autoantibodies to the proteins.

11. The diagnostic kit of claim 10 wherein the means for assessing the level of binding comprises a plate having wells wherein a first part of the wells are coated with the reference protein, preferably wild type TG2 or TG6, and a second part of the wells are coated with the at least one test protein, preferably at least one mutant TG2 or TG6.
12. A diagnostic kit for the diagnosis of a gluten-induced autoimmune disease in a subject comprising
a) a reference protein belonging to the transglutaminase family, said protein having an intact main
celiac epitope, wherein the core domain of said reference protein has a folded three dimensional
structure, and/or a medium carrying instructions for providing and using said reference protein
belonging to the transglutaminase family, and
b) a test compound known to be capable of binding to the main celiac epitope of said reference
protein belonging to the transglutaminase family, said main celiac epitope at least partially formed or
contributed by

one or more surface amino acid residue(s) of the first alpha helix of the core domain and/or
one or more surface amino acid residue(s) of the first alpha helix of the beta-sandwich domain
and of the conserved HisHisThr motif of the beta sandwich domain;

and optionally
means for taking or processing a biological sample from a subject, said sample containing
autoantibodies of said subject and/or
means for isolating autoantibodies from said sample, and/or
means for assessing the level of binding and/or kinetics of the test compound to the reference protein.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 9
Figure 9
Correlation of celiac antibody binding to the Ca-binding mutants
Figure 11
Figure 12
Figure 13

<table>
<thead>
<tr>
<th>slopes</th>
</tr>
</thead>
</table>
| S4     | -0.7079  
| Wt     | -0.4931  

Dissociation of cellac IgA

slopes:

- S4: -0.7079
- Wt: -0.4931
Figure 14